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Analysis of intestinal factors contributing to the age-dependency of systemic neuropathogenic *Escherichia coli* K1 infection in the neonatal rat

Thesis submitted in accordance with the requirements of the UCL School of Pharmacy for the degree of Doctor of Philosophy

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July 2012

PLAGIARISM STATEMENT

This thesis describes research conducted in the UCL School of Pharmacy between October 2008 and July 2012 under the supervision of Professor Peter W. Taylor. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of the dissertation that has already appeared in publication.

Signature: _____ Date: _____

Acknowledgements

Firstly I wish to thank my supervisor, Professor Peter Taylor, for giving me the opportunity to work on such an interesting and rewarding project. Your continued support and enthusiasm has been a constant source of encouragement and I greatly appreciate all the advice and help (both scientific and general!) that you have provided over the last four years. I owe you a lot of beer.

I also wish to thank my amazing parents for all their love and support over the eight years of my higher education. Without your enthusiasm and belief I would not have been able to follow this path. I sincerely promise I will now get a job!

Furthermore, I wish to thank colleagues at the London School of Hygiene & Tropical Medicine, Dr. Richard Stabler for all the help with the SSU arrays and Dr. Ozan Gundogdu and Melissa Martin for assistance with the gene expression arrays.

Finally, I am also very grateful to all members, past and present, of the Microbiology Group who have made the last four years such an enjoyable experience. Thank you Patri, Helena, Joao, Dave, Sarah, Christina, Lucia and Fatosh for all the great times, for all the advice, and for being guinea pigs for my JCR experiments! I would also like to extend my thanks to all the staff and students at the UCL School of Pharmacy who have made the institution such a friendly working environment. I wish you all the best for the future.

Dedicated to the memory of Charlie

Abstract

Systemic infections by encapsulated bacteria are a major aetiological agent of neonatal mortality. Neonatal meningitic *Escherichia coli* (NMEC) are isolated in a significant proportion of these infections. 80-85% of NMEC isolates express the K1 polysaccharide capsular antigen, a homopolymer of α-2,8-linked polysialic acid (PSA) which mimics the PSA modulator of neuronal plasticity in mammalian hosts and enables these strains to evade components of the innate and adaptive neonatal immune system. Systemic *E. coli* K1 infection is age-dependent. The basis of age-dependency is the capacity of the pathogen to translocate from the gastrointestinal (GI) tract into the systemic circulation. This initial step in pathogenesis is poorly characterized and the mechanistic basis of age-dependency is unknown. *Post-partum* development of the GI microbial population (microbiota) and host tissue may modulate susceptibility to *E. coli* K1.

Age-dependency was characterized in the neonatal rat model of infection. Two-day old (P2) neonates were highly susceptible to infection after oral dosing with *E. coli* K1 strain A192PP, whereas P9 neonates were highly refractive. This variation was not caused by the capacity of the pathogen to colonize the GI tract. The P2-P9 GI microbiota was assessed using culture-independent methods. Quantitative and qualitative analysis of the microbiota revealed that the P2-P9 microbiota was significantly different to that of the adult, but that very little variation occurred between the neonatal groups examined. Suppression of the P9 microbiota using combined antibiotic treatment did not increase the susceptibility of this group to *E. coli* K1. The P2-P9 development of the GI tissues and the response of P2 and P9 tissues to *E. coli* K1 colonization were assessed at the transcriptional level. A substantial degree of developmental expression was observed over P2-P9, including the up-regulation of putative components of the small intestinal (α-defensin peptides Defa24 and Defa-rs1) and colonic (trefoil factor peptide Tff2) mucus barrier. Colonization with *E. coli* K1 modulated expression of these peptides: The developmental expression of Tff2 was dysregulated in P2 tissues, likely due to IL-1β and NFκB signalling, and was accompanied by a decrease in the gel-forming mucin Muc2. Conversely, α-defensin expression was up-regulated in P9 tissues.

These results indicated that the intestinal barrier function of the P9 GI tract is more developed than the P2 equivalent. Furthermore, *E. coli* K1 colonization may compromise the development of the colonic mucus barrier in P2 neonates. This supports the hypothesis that the developmental state of the GI tissue, but not the microbiota, modulates susceptibility to systemic *E. coli* K1 infection. In addition, these results imply that supplementation of the neonatal GI with recombinant α-defensins or Tff2 represent potential strategies for the prophylaxis of neonatal *E. coli* K1 infection.

Table of Contents

Figures & Tables

Abbreviations

°C	Degree Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometre
AMP	Antimicrobial Peptide
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BCSFB	Blood-Cerebrospinal Fluid Barrier
BLAST	Basic Local Alignment Tool
BMEC	Brain Microvascular Endothelial Cell
bp	Base-Pair
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
C2BSC	Class II Biological Safety Cabinet
CFU	Colony Forming Unit
cm	centimetre
CM	Cytoplasmic Membrane
CNS	Central Nervous System
CR	Colonization Resistance
CSF	Cerebrospinal Fluid
DAEC	Diffusely Adherent *Escherichia coli*
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol

EAEC	Enteroaggerative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme-Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
EPEC	Enteropathogenic *Escherichia coli*
EONS	Early Onset Neonatal Sepsis
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extra-intestinal Pathogenic *Escherichia coli*
g	Gravity
g	Gram
GALT	Gut-Associated Lymphoid Tissue
GBS	Group B Streptococcus
gDNA	Genomic DNA
GF	Germ-Free
GI	Gastrointestinal
h	Hour
hCR	Host Colonization Resistance
HGT	Horizontal Gene Transfer
IgG	Immunoglobulin G
kb	Kilobase
kD	Kilodalton
KO	Knockout
L	Litre
LBP	Lipopolysaccharide Binding Protein
LOD	Limit of Detection
LONS	Late Onset Neonatal Sepsis

LPS	Lipopolysaccharide
M	Mole
mA	Milliamp
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
min	Minute
mL	Millilitre
mM	Millimolar
mCR	Microbiota Colonization Resistance
MODS	Multi-Organ Dysfunction Syndrome
NBM	Neonatal Bacterial Meningitis
NCAM	Neural Cell Adhesion Molecule
NCBI	National Centre for Biotechnology Information
NEC	Necrotizing Enterocolitis
NeuNAc	*N*-acetyl neuraminic acid
ng	Nanogram
NID	Non-Infectious Disease
NMEC	Neonatal Meningitic *Escherichia coli*
OD	Optical Density
OM	Outer Membrane
OMP	Outer Membrane Protein
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen-Associated Molecular Pattern
PAI	Pathogenicity Island
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit

pg	Picogram
PMN	Polymorphonuclear Leukocyte
PPG	Peptidoglycan
PRR	Pattern Recognition Receptor
PSA	Polysialic Acid
qRT-PCR	Quantitative Reverse Transcriptase PCR
RT-PCR	Reverse Transcriptase PCR
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulphate
sIgA	Secretory Immunoglobulin A
SIRS	Systemic Inflammatory Response Syndrome
SSU rDNA	Small-Subunit ribosomal DNA
T6SS	Type-VI Secretion System
TD	Thymus-Dependent
TFF	Trefoil Factor
TI	Thymus-Independent
TLR	Toll-Like Receptor
U	Enzyme Unit
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary Tract Infection
UV	Ultraviolet
V	Volt
VF	Virulence Factor
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

1.1 Infant mortality in the 21st century

1.1.1 Overview

In 2001 delegates gathered at the United Nations (UN) in New York for what was, at the time, the single largest meeting of world leaders in history. The purpose of the Millennium Summit was to discuss the role of the UN. in the new century and beyond, and resulted in the eight chapter Millennium Declaration, from which were derived the 8 Millennium Development Goals (MDGs) providing eight clear and achievable targets for global development to be met by 2015. The fourth MDG was targeted specifically at child health, with the goal of reducing by two thirds the mortality rates of infants (children under the age of five) compared to the 20% reduction observed over the previous decade (Figure 1.1).

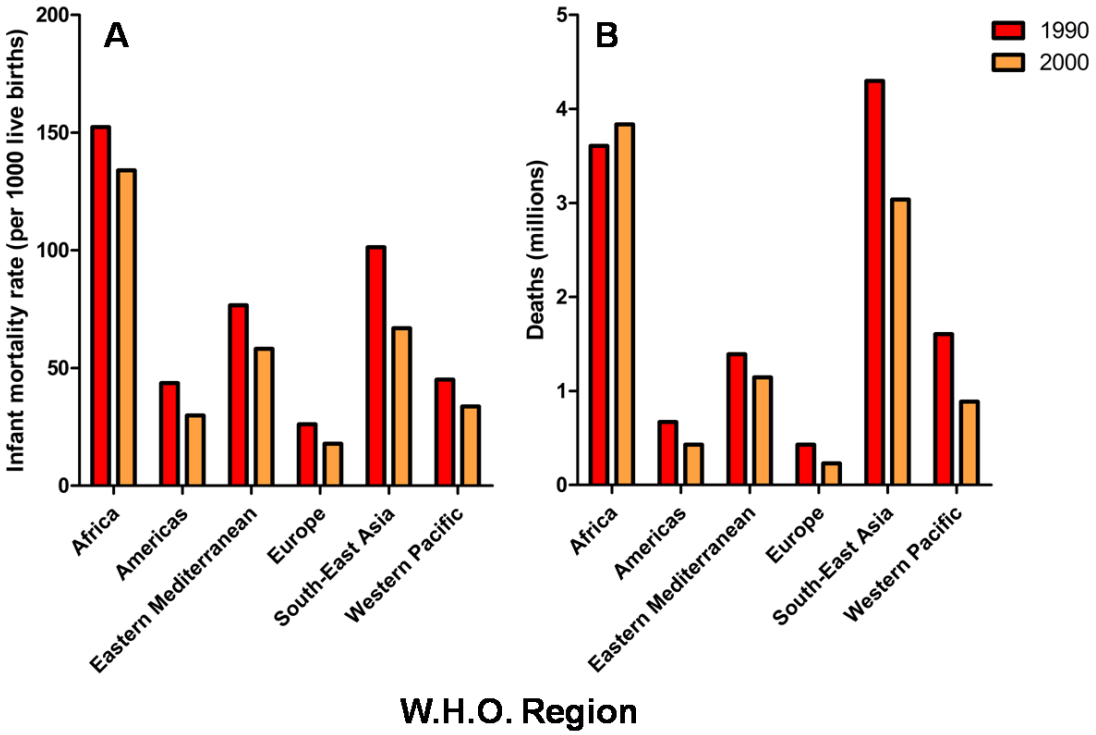


Figure 1.1: *Infant (children under 5 years old) mortality rates (A) and total deaths (B) recorded in the years 1990 and 2000. Data sourced from WHO Global Health Observatory Data Repository (http://apps.who.int/ghodata).*

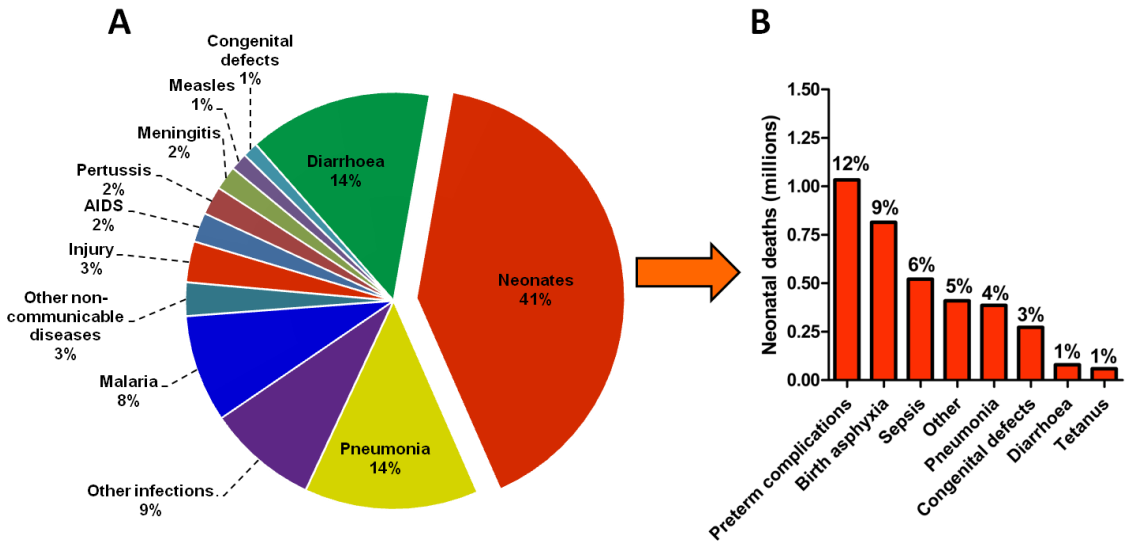


Figure 1.2: *Global causes of death for all infants under the age of five (A) and total deaths by cause of all neonates under the age of one month (B) in 2008. All percentages reflect proportion of total infant deaths (8.79 million) recorded in 2008. Data sourced from Black et al., 2010.*

The total number of infant deaths per year at the turn of the millennium was over 9.5 million, the equivalent of 26,000 deaths per day, or a nation with a population the size of Sweden, with the highest mortality rates and the vast majority of deaths occurring in the developing nations of the African and South East Asian regions.

Infant mortality has been attributed to multiple causes including fatal injuries, congenital defects, other non-communicable diseases and both preterm and *intrapartum* complications (Figure 1.2). However, as of 2008, the vast majority (64%) of infant deaths were directly attributable to infectious disease, with combined pneumonia, diarrhoeal disease, malaria and sepsis accounting for 77% of fatal infections. The available data also conclusively demonstrates that risk substantially decreases with age, with 77% of total infant mortality occurring in the first year of life and the most at-risk age group being the neonatal cohort, defined as infants less than one month old, which alone account for 41% (3.57 million in 2008) of all infant deaths (Black *et al.*, 2010).

1.1.2 The Increasing Importance of Neonatal Mortality

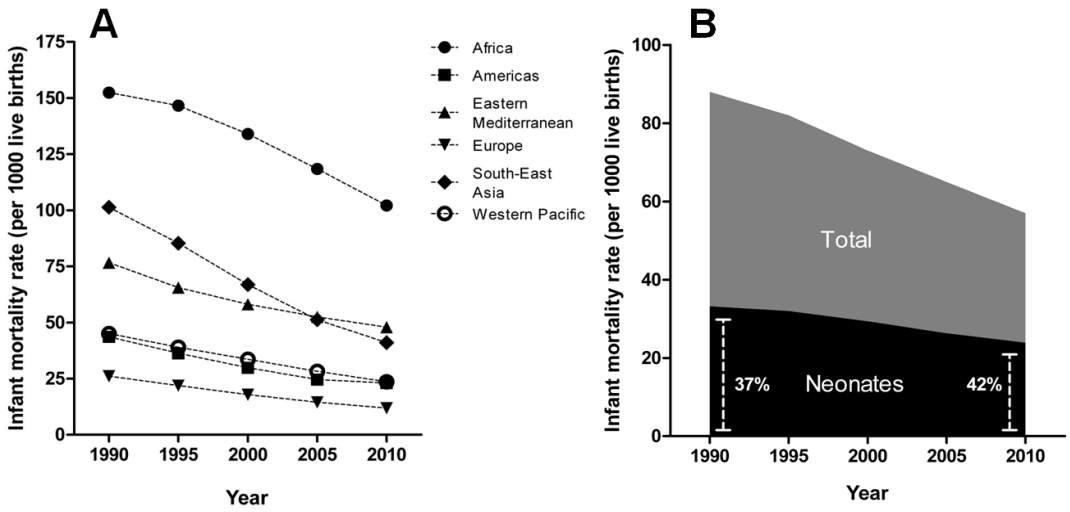


Figure 1.3: *Infant mortality rates from for each WHO region (A) and average global infant mortality rates (B) from 1990-2010 for all deaths occurring under the age of 5 years (Total) and under the age of one month 1 month (Neonates). Percentage neonatal mortality of total infant mortality for 1990 and 2010 are indicated in B. Infant mortality rate data sourced from WHO Global Health Observatory Data Repository (http://apps.who.int/ghodata), and neonatal mortality rate data sourced from Oestergaard et al., 2011.*

The relative importance, in terms of infant mortality, of the neonatal cohort has increased substantially in comparison to older infants since the initiation of the MDG program (Figure 1.3). According to the Inter-agency Group for Child Mortality Estimation (IGME) report "Levels & Trends in Child Mortality" published in 2011, the primary reason for this increase is that, although global infant mortality rates have declined by approximately 35% over the 1990-2010 period, with notable decreases in certain African and Asian regions, neonatal mortality has only declined by 28% over the same period. This equates to 1.7% per year, a significantly slower rate than the 2.2% per year decrease observed in the total infant mortality rate. This disparity in reduction rates has resulted in neonatal mortality accounting for up to 42% of the total infant mortality rate, a relative increase of over 10% from the 37% observed in 1990. The report also notes that the vast majority of neonatal mortality occurs in geographically restricted regions, with Sub-Saharan Africa and the Indian subcontinent combined accounting for

approximately two-thirds of all neonatal deaths worldwide. The disparity between neonates and older infants is due to the varying degrees of success encountered in reducing cause-specific mortality in the infant population (Figure 1.4). Comparison of data from studies conducted in 2003 (Bryce *et al.*, 2005) and 2008 (Black *et al.*, 2010) show that in the older cohort significant reductions have been achieved in deaths caused by pneumonia (38.9%; 0.78 million fewer deaths per year), diarrhoea (31.7%; 0.57 million fewer deaths per year), and autoimmune deficiency syndrome (44.8%; 0.14 million fewer deaths per year). A comprehensive WHO drive to improve global vaccination against the Measles virus in Egypt and Bangladesh, as part of an MDG orientated program, has also seen global immunization coverage expand from 74% in 2003 to 82% in 2008, resulting in an almost 80% decline in 2008 compared to 2003, the equivalent to 0.34 million fewer deaths per year.

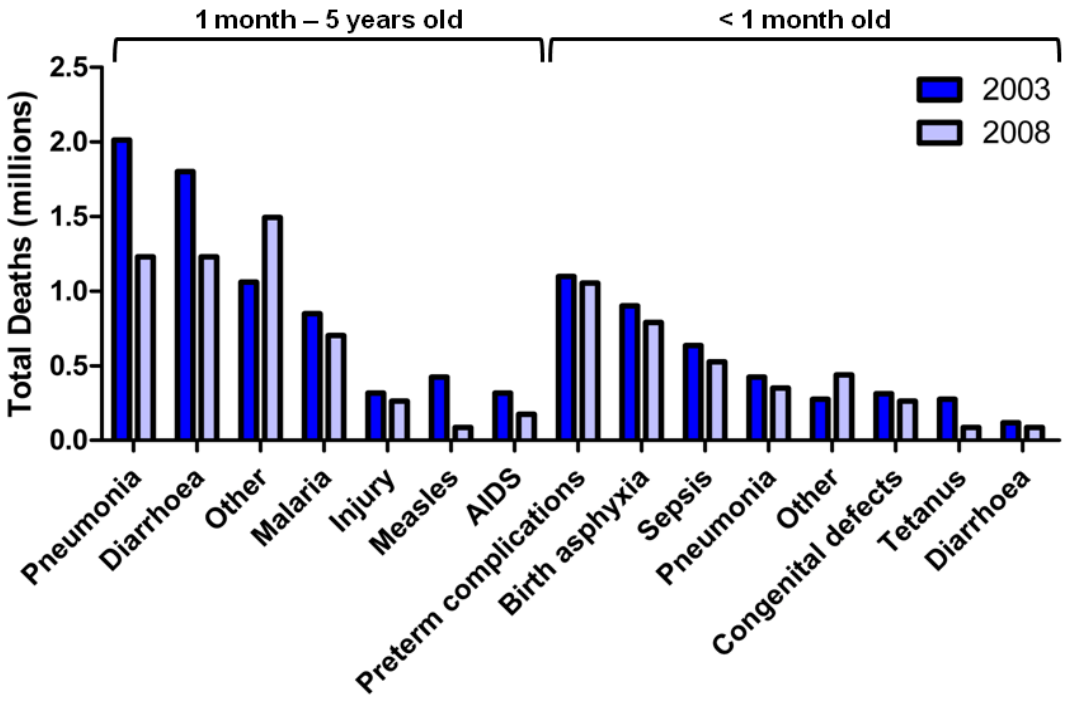


Figure 1.4: *Global total deaths, subdivided by cause, of infants from different age groups in 2003 (10.6 million deaths) and 2008 (8.79 million deaths). Data for different years sourced from Bryce et al., 2005 and Black et al., 2010 respectively.*

Although almost all of the primary causes of death in the neonatal cohort show reductions in deaths per year over this period, none of the causes which account for the majority of neonatal mortality (preterm and *intrapartum* complications, sepsis and pneumonia) decreased by more than 17%. An exception to this trend is that some

success has been achieved in the neonatal cohort by both maternal and neonatal immunization against the toxin produced by *Clostridium tetani*, the aetiological agent of tetanus, utilizing expanded distribution of the tetanus toxoid vaccine, with notable successes in Vietnam and other South-East Asia region nations. Overall this has resulted in a 68% decrease in neonatal tetanus deaths in 2008 compared to 2003, the equivalent 0.19 million fewer deaths per year.

1.1.3 Aetiology of Neonatal Mortality

The causes that result in neonatal mortality are partially distinct from those afflicting older infants. Some causes, by their nature, are clearly only applicable to either the neonatal or older infant cohorts, for example preterm and *intrapartum* complications (birth asphyxia) are major contributors to mortality and can only affect the neonatal cohort. Conversely neonates are much less susceptible to mortality induced by microorganisms which require over 30 days of incubation time prior to the development of lethal symptoms. Illustrative examples are the replication cycle of the malarial parasite *Plasmodium falciparum* in liver hepatocytes, prior to the infection of erythrocytes leading to the hemorrhagic complications associated with malarial mortality (reviewed by Miller *et al.*,1994) and the progression of perinatally acquired Human Immunodeficiency Virus (HIV) infection (Scott *et al.*, 1989) which involves degradation of the systemic $CD4^+$ T-cell population prior to the onset of the potentially lethal secondary infections associated with autoimmune deficiency syndrome (reviewed by Hel *et al.*, 2006). It should be noted however that mortality induced by HIV/AIDS is almost certainly acquired at the neonatal stage by maternal vertical transmission, emphasising the importance of the neonatal stage in the mortality of older infants. Broadly speaking, neonatal mortality can be subdivided into two aetiological groups, mortality induced by either non-infectious or infectious disease.

1.1.3.1 Non-infectious disease

Non-infectious diseases (NIDs) are responsible for a significant fraction of neonatal mortality with *intrapartum* complications, preterm complications and congenital defects attributed to 58% of neonatal deaths in 2008 (Black *et al.*, 2010). NID's are defined as conditions that are not directly caused by a pathogenic agent and hence cannot be transmitted from one individual to another. NIDs may account for a larger share of neonatal mortality than their infectious counterparts; however, there are microbial elements to the aetiology of all the major non-infectious causes of neonatal mortality. Both perinatal hypoxia (birth asphyxia) and the induction of preterm labour are strongly associated with intra-uterine infections (reviewed by Romero *et al.*, 2007; Goldenberg *et al.*, 2000) and maternal bacterial vaginosis also appears to be a significant risk factor in the development of pre-term labour (Hillier *et al.*, 1995), although whether or not this is simply as a marker of intra-uterine infection rather than a primary cause itself remains to be determined.

What is known is that one of the possible major complications of pre-term neonates, necrotizing enterocolitis (NEC), is mediated by microbial colonization of the neonatal intestines (reviewed by Morowitz *et al.*, 2010), with colonization by members of the Gram-negative Enterobacteriaceae family believed to be of direct significance in the development of the condition (Hoy *et al.*, 2000). Congenital infection of the foetus can also have a direct impact on the subsequent development of congenital defects (Epps *et al.*, 1995).

The microbes that can mediate these clinical outcomes are not restricted to a specific taxonomic grouping and contain representatives from the protozoan, bacterial and viral lineages (summarized in Figure 1.5). This serves as persuasive evidence that that the action of microbes, both directly and indirectly, plays a significant role in the aetiology of NID-related mortality, and should therefore be considered as potentially involved in all aspects of neonatal mortality and not solely in infectious disease.

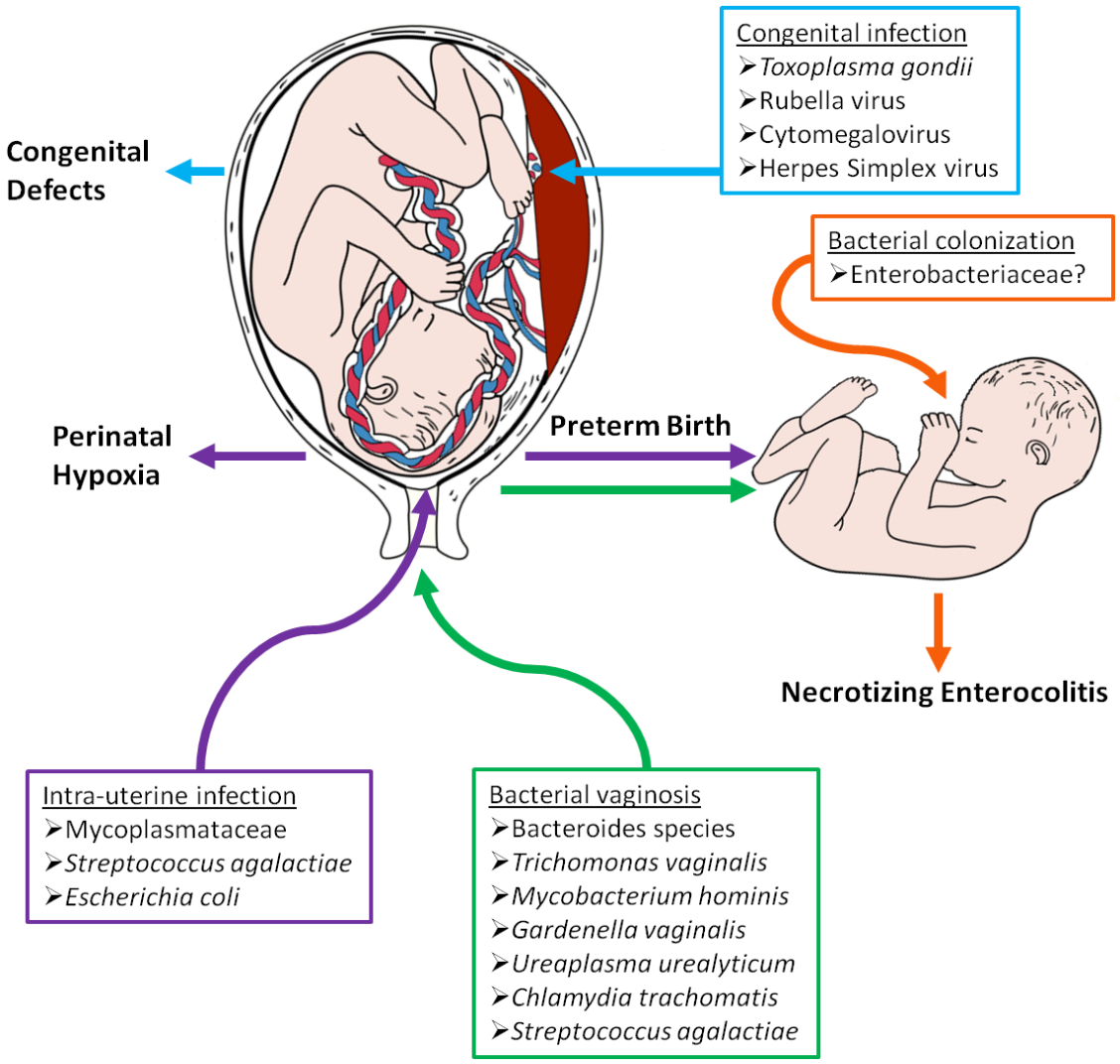


Figure 1.5: *Summary of microbial species and groups implicated in intra-uterine infection (purple), bacterial vaginosis (green), congenital infection (blue), bacterial colonization (orange) and the clinical outcomes associated with each grouping (bold). Image adapted from Wikimedia Commons file (Placenta.svg).*

1.1.3.2 Infectious Disease

Infectious diseases are aetiologically accountable for the remainder of neonatal mortality that is not covered by the aforementioned NIDs, and are thus responsible for approximately 42% of neonatal mortality. Infectious diseases differ from NIDs inasmuch as they are illnesses caused by infection with either a primary or opportunistic pathogenic agent (or agents), the pathogenesis of which directly mediates the clinical

symptoms of the disease. Although a range of infectious diseases can afflict the neonate, over 70% of deaths are due to four specific conditions, namely pneumonia, sepsis, diarrhoea and tetanus. Despite the greater overall burden of pneumonia as an infectious aetiological agent of mortality, in relation to neonates the single most prolific killer is sepsis, accounting for the bulk (35%) of all neonatal deaths with an infectious aetiology. This represents 15% of total infant deaths, the equivalent of over 527,000 deaths in 2008 (Black *et al.*, 2010).

1.1.3.2.1 Sepsis

Despite its prominence sepsis is problematic to universally define, with signs and symptoms varying between patients to such a degree that describing a 'typical' sepsis case remains a challenge (reviewed by Martinot *et al.*, 1997). However it is generally agreed that sepsis is the combination of two physiological events. Firstly infection itself, defined as the presence of microorganisms in normally sterile host tissues or fluids, and secondly the development of systemic inflammatory response syndrome (SIRS), defined as a combination of abnormal temperature, heart rate, respiratory rate and leukocyte blood cell count. Bacterial sepsis, the most common form, occurs when infecting viable bacteria penetrate the circulatory system and disseminate to systemic tissues haematogenously (bacteraemia). The infection can subsequently remain diffuse (septicaemia) or localize to multiple or individual organs leading to multi-organ dysfunction (MODS), pneumonia or meningitis.

Bacteraemia and the subsequent development of sepsis can occur at any age and carry a significant risk of mortality, especially in elderly patients with underlying medical conditions (Martin *et al.*, 2006). In relation to paediatric sepsis, the neonatal period presents the highest risk with mortality rates significantly higher than older children and survival directly related to the gestational age of infection, resulting in the status of preterm birth as a major mortality risk factor. A US-based multi-centre study of sepsis in children from 0-18 years of age found the highest rates (5.16 per 1000 live births) of sepsis in infants less than one year of age, and reported an overall case-mortality rate of 10.6% in this cohort (Watson *et al.*, 2003). Of these cases, 70% occurred in the neonatal period, and 60% of neonatal cases were preterm. Data from the

developing world is harder to assess but a review of community-based studies conducted in South Asian and African nations describes very high rates of neonatal sepsis ranging from 49-170 per 1000 live births, and case-mortality rates of up to 17% (Thaver & Zaidi, 2009). Both these measures are considered underestimates due to the lack of effective health system coverage in the developing regions in question.

In similar fashion to neonatal pneumonia, the microbial aetiology of neonatal sepsis is complex, with multiple bacterial species and groups represented. The microorganisms which are isolated from approximately 80% of sepsis patients are listed in Table 1.1. It is useful to distinguish two aetiological groups based on the timing of infection; early onset neonatal sepsis (EONS) is sepsis occurring in the first 72h post-partum and late onset neonatal sepsis (LONS) is sepsis which occurs after 72h but within the neonatal period of 30 days. Multi-centre studies of neonatal sepsis indicate that systemic infection with Gram-positive pathogens is responsible for the majority of both EONS (62%) and LONS (70%) cases in the US, with *Streptococcus agalactiae* dominating EONS and coagulase-negative staphylococci most frequently isolated in LONS cases (Stoll *et al.*, 2011/2002a). Interestingly, a meta-analysis of studies conducted in developing regions indicates that this does not hold true in global regions which account for the bulk of sepsis cases (Zaidi *et al.*, 2009). Gram-negative pathogens were isolated in 58% of EONS whereas LONS cases were evenly split between the two groups, with *Klebsiella* species and *Escherichia coli* accounting for almost 40% of EONS infections. Although Gram-positive pathogens appear to dominate sepsis in industrialized nations such as the USA, this is not reflected in the neonatal mortality rate. Gram-negative infection resulted in 36% mortality in LONS cases compared to 11% for Gram-positive infection (Stoll *et al.*, 2002a). The two pathogens which rank highest for EONS cases, Gram-positive *S. agalactiae* and Gram-negative *Escherichia coli*, also have highly divergent mortality rates at 9% and 33% respectively (Stoll *et al.*, 2011). This pattern is repeated in developing countries, compounded by higher rates of Gram-negative infection which significantly add to the burden of neonatal deaths in these regions (reviewed by Vergnano *et al.*, 2005).

Region	Bacterial pathogen	EONS	LONS
Industrialized (USA)	Coagulase-negative staph.	0.8%	47.9%
	Streptococcus agalactiae	43%	2.3%
	Escherichia coli	29%	4.9%
	Staphylococcus aureus	2%	7.8%
	Enterococcus spp.	3%	3.3%
	Streptococcus viridans	5%	
	Klebsiella spp.		4.0%
	Haemophilus spp	3%	
	Pseudomonas spp.		2.7%
	Enterobacter spp.		2.5%
	Serratia spp.		2.2%
	Streptococcus pyogenes	2%	
Total		370 (89%)	1313 (78%)
Developing	*Klebsiella* spp.	26.4%	5.6%
	Staphylococcus aureus	17.3%	13.7%
	Streptococcus agalactiae	13.1%	11.5%
	Escherichia coli	12.6%	9.3%
	Salmonella spp	0.7%	13.3%
	Streptococcus pneumoniae	1.1%	12.3%
	Streptococcus pyogenes	0.4%	9.7%
	Pseudomonas spp.	5.9%	1.8%
	Enterococcus spp.	5.3%	0.8%
	Enterobacter spp.	3.6%	1.2%
	Haemophilus spp.	0.1%	2.0%
	Listeria monocytogenes	0.5%	
	Streptococcus viridans	0.4%	0.1%
Total		834 (86%)	835 (81%)

Table 1.1: *Bacterial pathogens isolated from cases of early onset neonatal sepsis (EONS) and late onset neonatal sepsis (LONS) in industrialized and developing regions. Values represent % of total isolates. Totals represent number of sepsis cases examined and % coverage by the pathogens listed. EONS and LONS data for the industrialized regions sourced from Stoll et al., 2011/2002a respectively. Data for the developing regions sourced from Zaidi et al., 2009.*

Much as the signs and symptoms of sepsis are hard to define the characterization of its pathogenesis is complex. This is due to the multiple interrelated variables that interact to produce the ultimate bacteraemic state which typifies sepsis. The first variable to consider is the source of the pathogen. EONS pathogens are common constituents of the vaginal and gastrointestinal microbiota and are thus considered to be intrapartally acquired from the maternal microbiota. Conversely LONS pathogens are ubiquitous environmental organisms and constituents of the skin microbiota, and are thus thought to be nosocomially- or community-acquired. The second and third variables are the site of pathogen colonization and the invasive steps required to penetrate the host tissues and access the blood compartment, both of which are highly dependent on the specific pathogen encountered. The mucosal epithelial surfaces of the gastrointestinal and urogenital tracts, respiratory system, and oronasopharynx are colonized by a diverse range of commensal microorganisms, and are also common sites of initial pathogen colonization. Alternatively, physical disruption of the skin barrier function by injury or an indwelling catheter may provide direct access to the blood compartment for environmental pathogens. Persistent colonization of mucosal surfaces and the initial steps in epithelial translocation are mediated in part by bacterial adherence factors; examples include multimeric pilus structures such as type I pili (Fim proteins) from *E. coli* (reviewed by Schilling *et al.*, 2001) and RlrA from *Streptococcus pneumoniae* (Barocchi *et al.*, 2006), anchorless extracellular matrix-binding adhesins such as streptococcal PavA (reviewed by Chhatwal, 2002) and the multiple cell surface adhesins (IsdA, ClfB, SdrC/D) of *Staphylococcus aureus* (Corrigan *et al.*, 2009).

Post-colonization, sepsis-causing pathogens have to translocate across the tissue epithelium in order to access the bloodstream. Multiple factors contribute to this invasive process; one example is the glycosaminoglycan-binding Hek protein which is thought to contribute to epithelial cell invasion in sepsis-causing *E. coli* pathotypes (Fagan & Smith, 2007). *S. agalactiae* is believed to penetrate tissues by secretion of toxins such as β-haemolysin/cytolysin (βh/c) which mediate cytolytic damage to epithelial cells, disrupting barrier function and opening the epithelial gateway to the blood compartment and systemic circulation (Hensler *et al.*, 2005).

The fourth variable in the pathogenesis of sepsis is the mechanism employed by the pathogen to allow it to multiply in the host circulatory system whilst evading host immune responses. Again, multiple mechanisms have been identified; *Staphylococcus*

aureus coats itself with host antibodies by using protein A to bind the Fc fragment of IgG which inhibits recognition of the pathogen by Fc-receptors on host phagocytes, one of several mechanisms the organism possesses that inhibit immune function (reviewed by Foster, 2005). A prevalent mechanism of immune evasion is the elaboration of a polysaccharide capsule by the pathogen. Such capsules can be produced by streptococci, *Staphylococcus aureus*, *Haemophilus influenzae* and *Escherichia coli* and exploit the relatively poor immunological response of the neonatal immune system to polysaccharide antigens (reviewed by Klouwenberg & Bont, 2008). Furthermore some pathogens, if phagocytosed, have evolved to survive and replicate inside phagocytic leukocytes (Sukumaran *et al.*, 2003).

The ultimate cause of sepsis-induced mortality can be considered as the final variable in its pathogenesis. The systemic dissemination of pathogenic bacteria and the accompanying host inflammatory responses can result in both localized and systemically defined clinical outcomes. Induction of SIRS can result in MODS; a failure in the regulation of host homeostasis that results in the sequential failure of multiple organs. Although a full understanding of the mechanisms which drive MODS has not yet been achieved, it has been known for some time that pro-inflammatory cytokines IL-1β and TNFα are involved, as well as cellular components of the immune response (reviewed by Brown *et al.*, 2006; Abraham & Singer, 2007). Pneumonia can be both a prelude to and an outcome of sepsis. Pathogens may penetrate the blood compartment *via* the lungs or disseminate to them after invasion at an alternative site, both of which may result in eventual respiratory failure. A further, potentially deadly localized complication of sepsis is bacterial meningitis, an inflammation of the meningeal membranes that protect the brain and spinal cord which occurs when blood-borne pathogens penetrate the CNS, and which is detailed in the next section.

Sepsis is a devastating disease with a complex pathogenesis, derived from the multiple pathogens which act as its aetiological agents and the systemic nature of the infection and it can readily result in the development of fatal complications if left untreated. The treatment recommended by the WHO for suspected sepsis is immediate application of a dual intravenous antibiotic therapy targeting both Gram-positive and Gram-negative pathogens with a combination of aminoglycoside and expanded-spectrum β-lactam antibiotics. However the trend towards increasing resistance to front-line antibiotics in neonatal pathogens in both the developed and developing world

(Hyde *et al.*, 2002; Alarcon *et al.*, 2004; Thaver *et al.*, 2009) means that this strategy will become less effective over time and in the long-term may fail to provide any therapeutic benefit. This trend necessitates alternative treatment and prevention strategies, and a significant volume of research has been conducted in this area. This is typified by attempts at immunomodulation by transfusion of granulocytes, granulocyte growth factors and immunoglobulins in an endeavour to boost deficiencies in neonatal immune function that are thought to underpin the susceptibility of this age-group to sepsis. However, the results of multiple trials utilizing these techniques have so far failed to yield a significant decline in sepsis mortality (reviewed by Wynn *et al.*, 2009), but there is hope that a greater understanding of the early stages of sepsis pathogenesis and the maturation processes which govern the neonatal immune system may result in more positive results in the future.

1.1.3.2.2 Bacterial Meningitis

The WHO epidemiological neonatal mortality data published for 2003 and 2008 (Bryce *et al.*, 2005; Black *et al.*, 2010) does not list bacterial meningitis as a specific aetiology of neonatal mortality, with deaths from meningitis grouped with sepsis; however, this should not detract from the impact of this acute condition. The symptoms of meningitis in the neonate, unlike pneumonia, are indistinguishable from non-focal neonatal sepsis, and accurate diagnosis of meningitis is entirely dependent upon analysis of cerebrospinal fluid (CSF) sampled by a lumbar puncture procedure in order to detect any microbial pathogens which may have penetrated the CNS (Garges *et al.*, 2006). Although a relatively simple procedure, lumbar punctures are often not performed on preterm neonates due to the perceived risks of doing so, despite the fact that this neonatal group is at an elevated risk of meningitis, with the consequence that the condition is believed to be significantly underdiagnosed (Stoll *et al.*, 2004). Neonatal meningitis is diagnosed in a fraction of neonatal sepsis cases, with studies indicating that somewhere between 10-25% of reported sepsis cases progress to meningitis (Greenberg *et al.*, 1997; Sáez-Llorens & McCracken, 2003; Thaver & Zaidi, 2009). If concerns regarding the diagnosis of meningitis are correct, this proportion may be an underestimate. What is clear, however, is that the mortality rate of this condition is particularly high, especially in the developing world. Mortality rates in the developed

world have declined from 50% in the mid-20th century to approximately 10% at present (Puopolo *et al.*, 2005), however, despite this improvement the rate of morbidity has not declined to the same extent, with almost 20% of meningitis survivors afflicted with permanent severe or moderate neurological disabilities (Bedford *et al.*, 2001). Mortality rates in the developing world are poorly reported, however, a systematic review of 22 studies reported a median mortality rate of 40% (Furyk *et al.*, 2011).

Meningitis is a potentially lethal acute inflammatory condition which affects the meninges, the three membranes which envelop and protect the CNS. In order to understand meningitis it is first necessary to comprehend the structure and function of these membranes, and associated anatomical features, which are illustrated in Figure 1.6. The outermost meningeal membrane is the *dura mater*, the thickest and most structurally robust of the meninges, which is composed of fibroblast-like cells, a dense web of extracellular collagen fibres which provide its strength and elements of the cranial vasculature (reviewed by Adeeb *et al.*, 2012). The peripheral side of the *dura mater* is connected to the skull. The central meningeal membrane is the arachnoid, a multilayered but very thin epithelium with intercellular tight junctions and extracellular connections to both the *dura mater* and the innermost membrane, the *pia mater*. The *pia mater* is also extremely thin, intimately connected to the cerebral cortex by the extrusions of astrocytes and contains the cerebral vasculature which feeds blood into the cerebral cortex (Nakazawa & Ishikawa, 1998). The *pia mater* has several functions, including the formation of a perivascular space between the brain parenchyma and penetrating blood vessels, providing the organ with a form of lymphatic system (Zhang & Weller, 1990). The cavities between each meningeal membrane are the subdural and subarachnoid spaces which are filled with CSF, a vital component of the CNS which cushions the brain against concussive physical impacts and washes over the cerebral parenchyma, *via* the perivascular space, transporting nutrients to neurons and flushing metabolic waste back towards the circulatory system (reviewed by Cutler & Spertell, 1982). The CSF is produced in the four choroid plexi, specialized structures of the brain ventricles containing capillaries with fenestrated endothelia and the specialised ependymal cells of the choroid plexus epithelium which possess a range of apical ion cotransporters which actively transport Na^{+}, K^{+}, and Cl^{2-} into the CSF-containing ventricular lumen (reviewed by Wolburg & Paulus, 2010). This builds up a strong osmotic gradient between the blood and the CSF which precipitates water flux from the

circulation into the ventricles *via* a transcellular transport process across the epithelial cells mediated by the water channel protein AQP1 (Praetorius & Nielson, 2006).

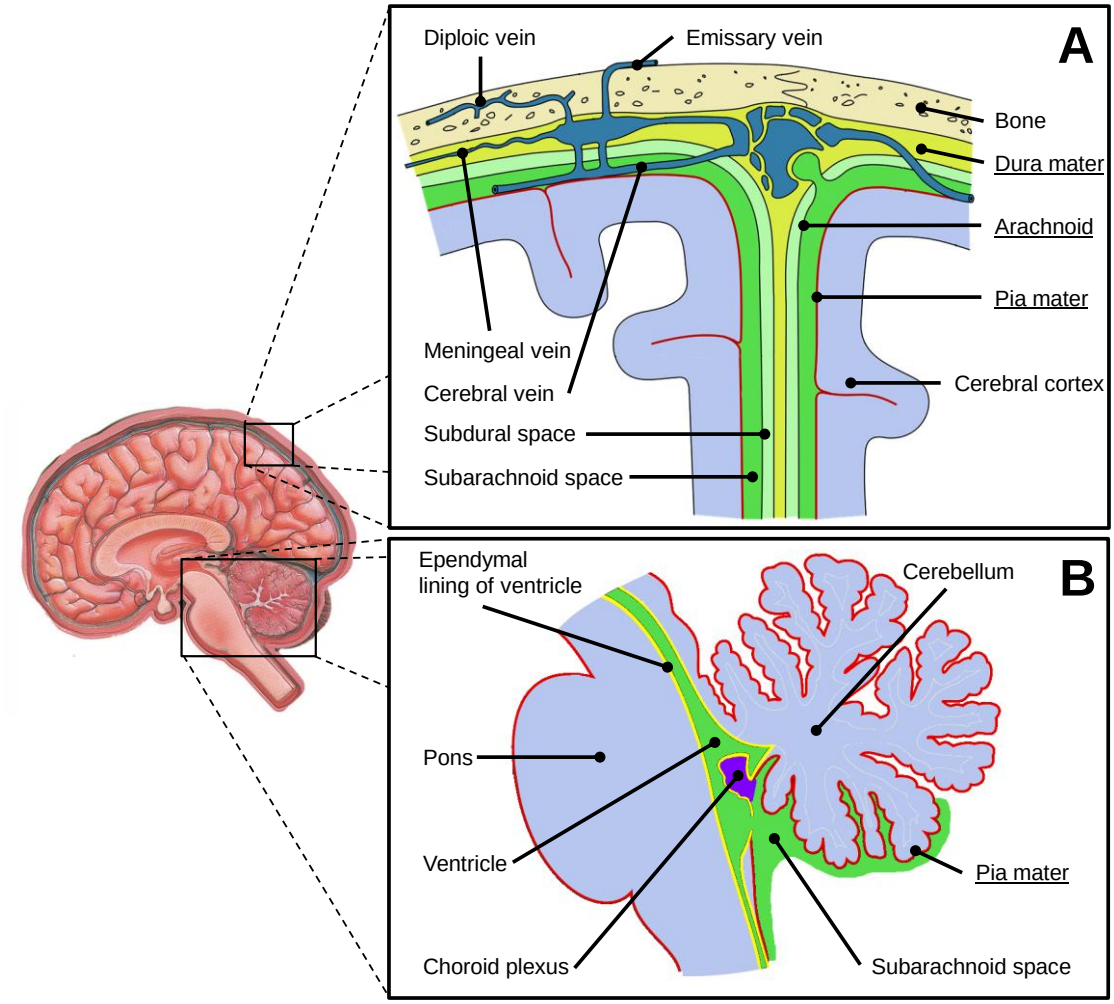


Figure 1.6: *Anatomy of (A) the meninges and associated neural, skeletal and vascular cranial structures; (B) the choroid plexus and surrounding tissues. The three meningeal membranes are underlined. Images adapted from Wikimedia Commons files (Gray769.png; Gray708.svg).*

Meningitis frequently occurs as a sequela of bacteraemia and sepsis with bacterial pathogens gaining access by various mechanisms and potential routes to the CSF compartments *via* the circulatory system. Bacteria subsequently propagate throughout the CSF, spreading through the subarachnoid and subdural spaces. Despite the fact that a significant fraction of meningitis-causing bacteria are encapsulated,

inhibiting recognition by elements of the immune system, bacterial replication and lysis releases prokaryotic cellular components into the CSF. Components that have been implicated in the pathophysiology of meningitis include classical stimulators of the inflammatory response such as the Gram-positive cell wall constituents peptidoglycan (PPG) and lipoteichoic acid (LTA) and the Gram-negative outer membrane-bound LPS-containing endotoxin complex (Tuomanen *et al.*, 1985; Syrogiannopoulos *et al.*, 1988). The fact that bacterial debris modulates the inflammatory response in the CNS is evidenced by the fact that bactericidal antibiotic treatment of meningeal infections results in increased release of these products and a correlating increase in inflammation (Mertsola *et al.*, 1989; Arditi *et al.*, 1989).

Until relatively recently the CNS was regarded as an 'immunologically privileged' site with a relatively immunoincompetent leukocyte population composed of microglia cells and hidden from the adaptive lymphocyte-driven immune response by its isolation behind the endothelial blood-brain barrier (BBB). This view has been challenged by studies demonstrating that, although both innate and adaptive immune responses are differentially regulated in the CNS, they do interact with the peripheral immune system. The BBB is permeable to leukocytes and lymphocytes and the peripheral microenvironments of the CNS, namely the meninges and sub-meningeal spaces, have populations of highly immunocompetent macrophage and dendritic cells capable of stimulating robust innate and adaptive immune responses (reviewed by Carson *et al.*, 2006).

Innate immune response pathways are stimulated in these leukocytes by the aforementioned prokaryotic cellular components, which are pathogen-associated molecular patterns (PAMPs). For example, LPS is complexed by extracellular LPS-binding protein (LBP) which is then recognized by the pattern-recognition receptor (PRR) proteins CD14 and TLR4 (Poltorak *et al.*, 1998; Muta & Takeshige, 2001). PPG and LTA are recognized by a heterodimer of two Toll-like receptors, namely TLR2 and TLR6 (Takeuchi *et al.*, 1999; Ozinsky *et al.*, 2000). Binding to, and activation of, these PRRs results in activation of primary transcription factors such as NFκB (reviewed by Gilmore, 2006), leading to the production of pro-inflammatory cytokines that include TNFα, IL-1β, IL-6, IL-8, and platelet-activating factor (PAF), which are commonly detected in increased quantities in CSF samples from clinical meningitis cases (Ramilo *et al.*, 1990; reviewed by Sáez-Llorens *et al.*, 1990).

Secretion of pro-inflammatory cytokines by meningeal monocyte-derived leukocytes induces expression of cellular adhesion molecules ICAM1 and VCAM1 in brain vascular endothelial cells. The cytokine chemotactic gradient attracts circulating innate-effector leukocytes such as neutrophils which bind to the aforementioned adhesion molecules and translocate into the sub-meningeal spaces (Henninger *et al.*, 1997; Bohatschek *et al.*, 2001). The migration of circulatory leukocytes into the inter-meningeal spaces represents the inflammatory process that is the critical step in the pathophysiology of meningitis. As with other inflammatory conditions, such as pneumonia, the vasogenic influx of leukocytes stimulated by macrophage and dendritic cells is vital in both limiting and combating infection in the meninges (Polfliet *et al.*, 2001). However the deleterious cytotoxic effects of inflammation, such as the production of reactive oxygen species and nitric oxide, and increased permeability of the BBB during vasogenic influx can have significant consequences, potentiating the development of lethal sequelae. These include oedema, hypertension, and decreased blood flow to the brain parenchyma (Tauber, 1989; Koedel *et al.*, 1995), leading to hypoxia, neuronal apoptosis and eventual death.

As a potential consequence of sepsis, the aetiology of neonatal bacterial meningitis is represented by a restricted cohort of the pathogens that comprise the aetiological agents of EONS and LONS. As with these conditions, there are some regional variations with respect to the pathogens isolated in developed and developing nations. A multi-centre study of neonatal sepsis and meningitis in the US reported an equal number of Gram-positive and Gram-negative meningitis cases, with *E. coli* accounting for 44% and *S. agalactiae* 19% of total meningeal infections (Stoll *et al.*, 2011). As expected, data from developing nations is much harder to evaluate; however, a recent systematic review of 22 reports with representative studies from most geographical regions of the developing world appears to indicate that *E. coli*, *S. agalactiae*, *Klebsiella* spp. and *S. pneumoniae* are the four most frequently isolated pathogens (Furyk *et al.*, 2011). All of these pathogens, and thus the bulk of meningitis isolates, express capsular polysaccharide.

The polysaccharide capsule is an essential virulence factor in relation to both Gram-positive and Gram-negative bacterial neonatal pathogens with regard to their capacity to cause meningitis. The molecular composition of the polysaccharide can be that of a homopolymer, consisting of a single repeated monosaccharide, or a

heteropolymer comprised of repeating units of 2-6 different sugar monomers. The primary function of the capsule is defensive, with the long polysaccharide chains masking the bacterial cell from potentially hostile determinants, including cellular and humoral elements of the neonatal immune system (Kolb-Maurer *et al.*, 2001; reviewed by Moxon & Kroll, 1990). The neonatal humoral immune system performs particularly poorly in the recognition of foreign polysaccharide, as it constitutes a thymus-independent type 2 antigen (reviewed by Weintraub, 2003; Klouwenberg & Bont, 2008). Antigens can be broadly classified as thymus-dependent (TD) or thymus-independent type 1 or 2 (TI-1 or TI-2) based on whether the immunological response requires the involvement of thymus-derived $CD4^+$ T-cells or can be directly mediated by B-cells without T-cell involvement. Most proteins are TD antigens; LPS is an example of a TI-1 antigen and most polysaccharides, as indicated, are TI-2 antigens. TI-2 antigens were first differentiated from TI-1 antigens by the lack of response of neonatal B-cells to certain molecules, including polysaccharide (Mosier *et al.*, 1977), and it was later shown that responsiveness to TI-2 antigens in humans does not develop until 2 years of age, due to immature B-cell receptor deficiencies (reviewed by Rijkers *et al.*, 1998). This developmental deficiency is a key determinant in the susceptibility of neonates to infection by encapsulated pathogens; however, some meningitis-causing bacteria employ an extra layer of subterfuge in that their capsules mimic the molecular structure of a host antigen. A prime example of this capsule class is a homopolymer of α-2,8 linked N-acetyl neuraminic acid (NeuNAc), also termed polysialic acid (PSA), which is elaborated by *E. coli* capsular serotype K1 (*E. coli* K1) and *Neisseria meningitidis* capsular serotype B (Group B meningococcus). This structure mimics a host-derived PSA glycoconjugate, which functions as a key regulator of neuronal plasticity during neonatal cerebral development through its interactions with neural cell adhesion molecules (NCAM; reviewed by Rutishauser, 1996; Troy, 1992). This structure is extremely poorly immunogenic (Keller *et al.*, 1980; Jennings & Lugowski, 1981) and only appears to elicit an IgG-mediated immunological response in hosts with autoimmune hyper-reactivity (Frosch *et al.*, 1985).

Many clinically significant bacteria can produce capsular polysaccharide, with sub-strains of the same species capable of producing a diverse range of biochemically distinct structures, giving rise to multiple capsular serotypes. The number of capsular serotypes that have been identified for a given bacterial species is variable; however, the

species that are most frequently isolated in cases of neonatal meningitis tend to be among the most prolific in terms of capsular diversity with, over 80 different serotypes identified in *E. coli*, *Klebsiella* species and *S. pneumoniae* (reviewed by Weintraub, 2003; Whitfield, 2006; Ørskov & Ørskov 1984; Podschun & Ullmann, 1998). The exception is *S. agalactiae*, which has relatively few capsular serotypes, with only 9 identified at present (Ryc *et al.*, 1988; Slotved *et al.*, 2007). Although all capsules serve a defensive function, only a relatively restricted cohort are associated with neonatal invasive disease and meningitis. These include *E. coli* capsular serotypes K1, K2 and K5 (Korhonen *et al.*, 1985); *Klebsiella* capsular serotypes K1, K2, K4 and K5 (reviewed by Podschun & Ullmann, 1998); *S. pneumoniae* capsular serogroups 1, 19, 6, 5 and 14 (Hausdorff *et al.*, 2000) and *S. agalactiae* capsular serotype III (reviewed by Schuchat, 1998). The underlying cause of this association can be traced in part to additional factors relating to polysaccharide structure, such as molecular mimicry of host antigens (Troy, 1992; Vann *et al.*, 1981) and intrinsic resistance to specific innate immune mechanisms (Kabha *et al.*, 1995); however the basis of a good deal of specific capsular serotype-virulence relationships remains to be fully described.

As with its parent condition, sepsis, bacterial meningitis is clearly an aetiologically complex and potentially fatal complication of the neonate which requires prompt treatment in order to elude its associated morbidities and high mortality rate. The recommended treatment of meningitis mirrors that of sepsis, namely an aggressive antimicrobial chemotherapeutic strategy targeting both Gram-positive and Gram-negative bacteria, with the additional requirement that the agent in question be able to transverse the BBB into the CNS. However, as described in the previous section, antimicrobial resistance in neonatal pathogens is on the rise. A recent study of neonatal pathogen resistance patterns in the developing world has shown that the resistance of the two predominant Gram-negative aetiological agents of neonatal meningitis, *Klebsiella* and *E. coli*, to Ceftriaxone, a 3rd generation cephalosporin class β-lactam that is commonly used to treat meningitis, has risen from 33% to 66% and 0% to 19% respectively over the previous decade (Thaver *et al.*, 2009). This factor, combined with the high morbidity rates observed even after effective antimicrobial treatment, strongly indicates that new alternative treatments and prophylactic strategies should be employed in combating this disease.

1.1.4 Reducing Neonatal Mortality

The UN 2010 MDG report indicated that infant mortality significantly declined over the previous 2 decades, but did not fall fast enough to achieve the 4th MDG target of a global two-thirds reduction by 2015. Significant gains have been made in reducing mortality in infants, with dramatic decreases in mortality due to diarrhoea and pneumonia observed in infants outside the neonatal cohort. This success has been primarily due to the use of effective condition-specific management strategies such as oral rehydration therapy (ORT) in the case of diarrhoea and the WHO prescribed case-management approach in relation to pneumonia (Victoria *et al.*, 2000; Sazawal *et al.*, 2003; Theodoratou *et al.*, 2010a). These core strategies can be expected to continue to reduce infant mortality as their coverage expands further. Additionally, data suggests that supplemental reductions in mortality may well be achieved by expansion of vaccination programmes against the common viral and bacterial aetiological agents of these diseases (Jiang *et al.*, 2010; Simonsen *et al.*, 2011; Theodoratou *et al.*, 2010b). It appears that progress in these areas, whilst by no means complete at this stage, may feasibly result in these two global killers losing their pole position in terms of infant mortality in the not too distant future.

Unfortunately, these developments are not as advantageous to the neonatal cohort, where diarrhoea and pneumonia only accounted for 12% of neonatal mortality in 2008 (Black *et al.*, 2010). In order to have any hope of achieving drastic reductions in infant mortality, the afflictions of the neonatal cohort must be addressed as a matter of urgency. Over the course of the previous few sections, I have reviewed the aetiological basis of neonatal mortality and, from this, several themes have emerged. Firstly, although the two foremost afflictions of the neonate, preterm birth and intrapartum complications, are classed as NIDs the direct or indirect role of microorganisms in the development of these afflictions should not be understated. From the pathophysiology of preterm complications such as NEC and the intrauterine infections implicated in driving both preterm birth and perinatal hypoxia, as well as the more direct involvement of the pathogens isolated in infectious neonatal disease, it is clear that the focus of any strategy to reduce neonatal deaths must derive from a greater understanding of the relevant microorganisms and the pathogenic mechanisms that drive the aetiological motors of mortality.

Other themes to emerge are the role of immunological responses in the progression of neonatal disease and the role that inflammation plays in mediating mortality. The development of the various elements of the neonatal immune system is a rich area of research and not without controversy. Whilst some research indicates that adaptive immune pro-inflammatory responses are dulled in the neonate compared to the adult (reviewed by Levy, 2007) others, have reported over-production of inflammatory mediators in response to innate immune stimuli (Tatad *et al.*, 2007; Zhao *et al.*, 2008). Whether or not elements of the neonatal immune system are in some way impaired or hyper-responsive, prolonged and/or excessive inflammatory responses appear to inflict the bulk of the damage responsible for the mortality observed in sepsis, pneumonia and meningitis. Thus, a greater understanding of neonatal developmental immunology will allow the refinement or development of therapeutic and prophylactic strategies designed to compensate for immunodeficiencies in the developing neonate.

The third major theme is the conservation of specific microorganisms across the spectrum of neonatal disease. Several species recur in the microbial aetiology of neonatal disease, although *E. coli* and *S. agalactiae* are among the most consistently prominent, with both pathogens implicated in intra-uterine infections that can prompt preterm birth and perinatal hypoxia, as well as sepsis, pneumonia, meningitis, and, in the case of *E. coli*, diarrhoea. The fact that these microbes are constituents of the maternal gastrointestinal or vaginal microbiota explains why these pathogens dominate intra-uterine and early-onset forms of disease, as they are frequently among the first microorganisms encountered by the neonate.

S. agalactiae, commonly referred to as the group B streptococcus (GBS), was recognized as a prominent agent of neonatal mortality in industrialized countries in the 1970s and, as a result, the use of *intrapartum* antibiotic chemoprophylaxis was trialed and found to be effective in reducing the incidence of neonatal GBS infections and associated mortality (Boyer *et al.*, 1986). Developments throughout the 1990s led to recommendations for standardized culture-based GBS screening of pregnant women and antibiotic treatment (Halsey *et al.*, 1997), with the result that GBS disease has significantly declined in the neonatal population since their implementation (Brooks *et al.*, 2006). The use of *intrapartum* antibiotics has not reduced rates of neonatal *E. coli* infection (Schrag *et al.*, 2006) and has been accompanied by reports of an increasing

incidence of neonatal disease caused by this pathogen, especially in the preterm population (Stoll *et al.*, 2002b; Cordero *et al.*, 2004; Bizzarro *et al.*, 2008).

Although GBS screening is mostly confined to industrialized nations, the benefits of this prophylactic measure make it likely this procedure will be implemented in developing nations in the near future. Should the pattern of increased neonatal *Escherichia coli* infection observed in industrialized nations be observed in developing nations, this will increase the burden of disease caused by this pathogen in regions which already account for the majority of neonatal infectious disease mortality, and which already suffer from increased rates of Gram-negative bacterial infections and associated elevated mortality rate. Increased infections in industrialized nations, the relatively high rates of infection in developing nations and the potential for increases in these rates mean that effective treatment strategies for the management or prophylaxis of neonatal *Escherichia coli* infection are essential in order to reduce neonatal mortality. Although the resistance of GBS to frontline antibiotics does not appear to have significantly increased since the introduction of *intrapartum* chemoprophylaxis (Heelan *et al.*, 2004; Chohan *et al.*, 2006), the same does not hold true for *E. coli*, with isolates showing significant increases in resistance to multiple antibiotic classes (Hyde *et al.*, 2002; Thaver *et al.*, 2009). This strongly indicates that antibiotic therapy cannot alone reduce infections by this pathogen and efforts should be made to understand its pathogenesis, allowing specific steps on the path to mortality and morbidity to be therapeutically targeted.

1.2 *Escherichia coli*

1.2.1 Natural History

E coli belongs to the Enterobacteriaceae and was originally isolated in 1886 by the German paediatric bacteriologist Theodor Escherich following his investigations into bacteria that inhabited the infant colon. The bacterium is a Gram-negative rod-shaped non-sporulating facultative anaerobe approximately 2 µm long and 0.5 µm wide. *E. coli* is the most thoroughly characterized organism, with the best known strain being K-12, an isolate which has been grown in the laboratory for almost a century and from which a large number of mutant strains have been derived (Bachmann, 1972). Due to the relative ease and safety with which it is cultured and the numerous techniques that have been developed for its manipulation at the molecular level, this strain has long served as a model organism for a range of microbiological disciplines, including genetics, metabolism, proteomics and evolution and it was one of the first organisms to be genome sequenced (Blattner *et al.*, 1997). Some strains, such as protease-defective BL21, are widely used in the biotechnology industry as a recombinant microbiological system for the large scale production of prokaryotic and eukaryotic heterologous proteins (reviewed by Baneyx, 1999).

Outside of the laboratory, *Escherichia coli* is a principal component of the intestinal microbiota of infants (Penders *et al.*, 2005) and is also present to a lesser extent in the adult intestine, in which facultative anaerobes make up a much smaller proportion of the bacterial population (Eckburg *et al.*, 2005). The gastrointestinal (GI) tract of endothermic organisms is considered to be the natural habitat of *E. coli* and the bacterium has been used as a biological marker of faecal contamination as it was considered to be unable to survive for long outside the GI tract. However, several studies have demonstrated that under certain conditions *E. coli* can colonize and replicate in environments external to the host GI tract (Desmarais *et al.*, 2002; Ishii *et al.*, 2006; Liang *et al.*, 2011), demonstrating a surprising environmental versatility.

The ecological adaptability of *E. coli* may be explained by its versatility in key biological arenas, especially with regard to its metabolic capabilities. It is able to utilize

a wide range of carbon-containing compounds as sole source of carbon and for generation of adenosine tri-phosphate (ATP). The catabolic pathways that *E. coli* are capable of utilizing to form ATP from these sources are highly varied. As a facultative anaerobe, *E. coli* has the biochemical means to utilize both oxygen (aerobic) and fumarate or nitrate (anaerobic) as terminal electron acceptors in its respiratory ATP-generating chain (reviewed by Ingledew & Poole, 1984). Switching between these two respiratory pathways is regulated by the oxygen sensitive FNR global transcriptional regulator and its associated regulon (Constantinidou *et al.*, 2006). Additionally, in the absence of these electron acceptors and the presence of a suitable substrate, *E. coli* continues to produce ATP by mixed-acid fermentation (reviewed by Clark, 1989) although this, and the alternate forms of anaerobic respiration, are significantly less efficient in producing ATP than aerobic respiration.

E. coli are a Gram-negative species and have a cell wall structure typical for this group of bacteria (Figure 1.7). The cytoplasmic membrane (CM), a hydrophobic phospholipid bilayer containing an array of membrane associated proteins many of which function in an influx/efflux transporter capacity (Daley *et al.*, 2005) and mediate electron transport for the various ATP-generating pathways, retains vital metabolic components and nucleic acids within the cytoplasm. External to the CM is the periplasm which contains the sugar/amino acid heteropolymer peptidoglycan (PPG) and another phospholipid bilayer, the outer membrane (OM). The periplasm contains a continuous mesh of PPG which forms the sacculus, a cell-encompassing macromolecule which is synthesised in the CM (Bupp & van Heijenoort, 1992) and may be regulated by complexes of the cytoplasmic actin homologue MreB and membrane-bound RodZ (van den Ent *et al.*, 2010), although the exact role of these proteins has yet to be determined (Swulius *et al.*, 2011). The PPG sacculus is anchored to the OM by a murein lipoprotein (Braun & Sieglin, 1970) and provides rigidity and structure to the cell wall.

Beside the Braun murein lipoprotein, the OM proteins (OMPs) include iron receptors (FhuE, FhuA), porins (OmpC, OmpF) and the porin-like multifunctional high-copy β-barrel OmpA (Molloy *et al.*, 2000). Biogenesis of the OM is mediated by a complex of OMPs, including YeaT (Omp84), which is essential for the proper folding of other OMP proteins (Wu *et al.*, 2005). The OM bilayer has an outer leaflet composed of LPS (Kamio & Nikaido, 1976). The structure of LPS is illustrated in Figure 1.8 and

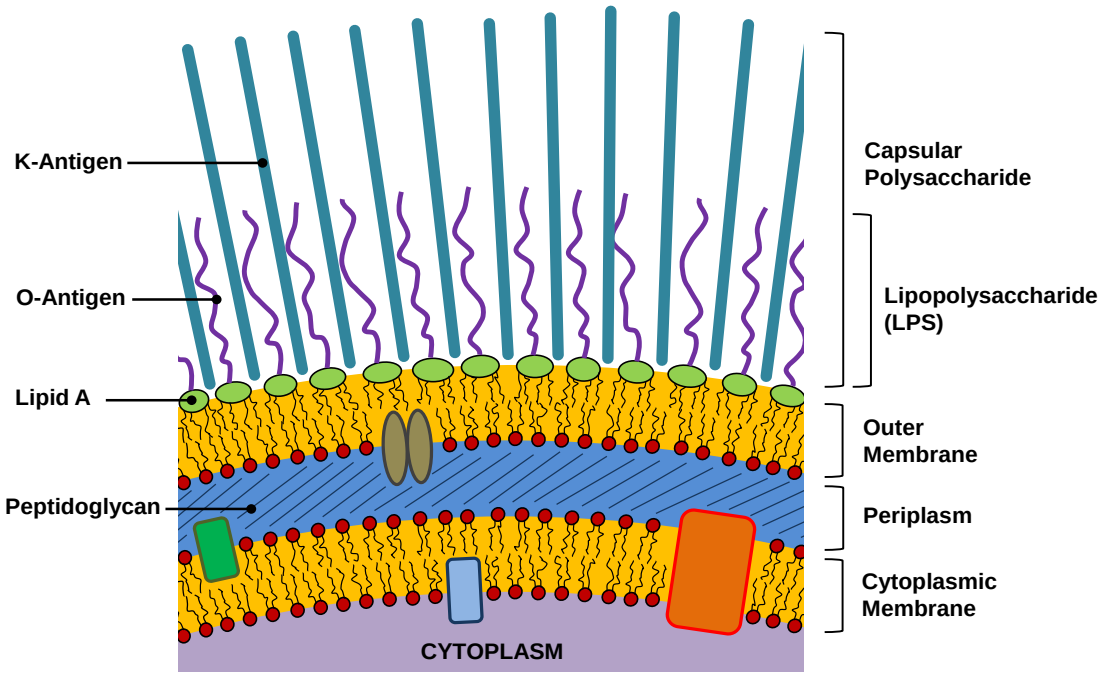


Figure 1.7: *The cell wall of an encapsulated Escherichia coli cell illustrating the membrane structure and external surface O-antigen (LPS) and K-antigen (capsule). H-antigen (flagellum) is not illustrated.*

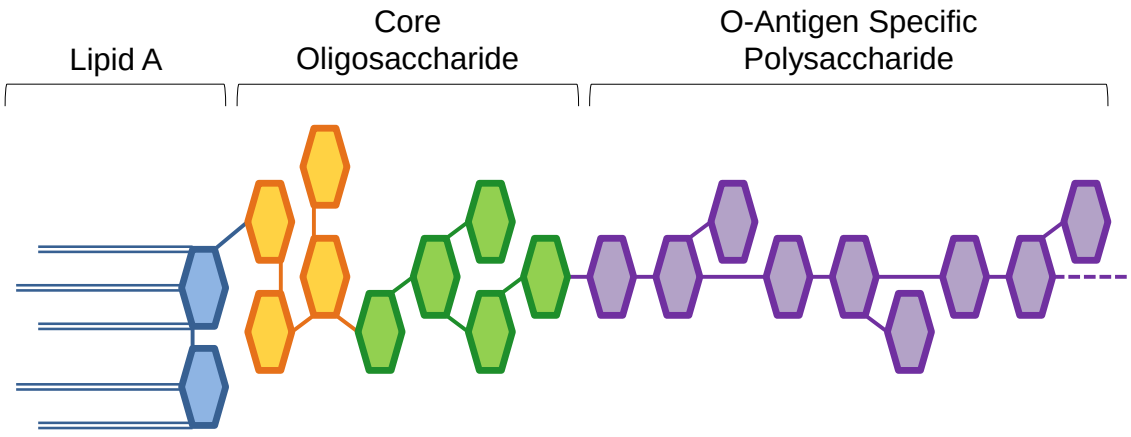


Figure 1.8: *Representation of lipopolysaccharide (LPS) components. Lipid A (blue), core oligosaccharide (inner: yellow; outer: green) and the O- Antigen serotype-specific polysaccharide (purple) are indicated.*

consists of the proximal membrane-anchored constituent lipid A, the inner and outer core oligosaccharide linked to lipid A and the distal O-Antigen polysaccharide. Lipid A is the endotoxin component of the molecule, which is recognised by the innate LBP/CD14/TLR4 receptor pathway, stimulating a strong immune response (Poltorak *et al.*, 1998; Muta & Takeshige, 2001) but which is also structurally essential to the *E. coli* cell (Galloway & Raetz, 1990). The inner core oligosaccharide is generally conserved within species but the outer core is more variable, with 5 different variants currently known of in *E. coli* (reviewed by Heinrichs *et al.*, 1998). The core does not appear to be vital to *E. coli* cellular viability but does appear to influence the stability of the outer membrane by the formation of intermolecular cationic bonds between core domains (reviewed by Vaara, 1992), as well as providing a linkage site for the O-antigen polysaccharide. The repeating oligosaccharide units that constitute the O-antigen polysaccharide are of great epidemiological significance to *Escherichia coli* as they are serologically heterogeneous, with over 170 different serotypes thus far identified within the species (reviewed by Raetz & Whitfield, 2002). If the strain is non-capsulated, the O-antigen is the peripheral component of the cell and has a protective function. The polysaccharide prevents the bactericidal and/or lytic actions of both the serum complement cascade and neutrophil-secreted BPI protein in a length-dependent fashion (Burns & Hull 1998; Weiss *et al.*, 1986). Both the toxic effect of lipid A and the innate immune-evasion function of the O-antigen mean that this component of the *E. coli* cell wall is generally considered to be a virulence factor in pathogenic strains of the organism.

If the *E. coli* strain is encapsulated, the capsular polysaccharide, or K-antigen, constitutes the outermost component of the cell. In similar fashion to the O-antigens, capsules are highly heterogeneous, with approximately 80 different serotypes thus far identified (reviewed by Weintraub, 2003; Whitfield, 2006). Serotyping of *E. coli* has been utilized since the 1940's, with serological characteristics and thermostability initially used to classify the K-antigens into 3 groups. The currently used system of Whitfield and Roberts breaks the different K-antigens down further into 4 groups based on genetic and biosynthetic criteria rather than structure (Whitfield & Roberts, 1999). Group 1 (e.g. K30) and 4 (e.g. K40) capsules are closely related to the O-antigen polysaccharide, with each K-antigen expressed as two distinct forms on the cell surface, one linked to the LPS lipid A-core (K_{LPS}) and the other which is not (MacLachlan *et al.*,

1993; Amor & Whitfield, 1997). In genetic terms, the *cps* gene clusters required for capsular expression for both groups are located near the *his* locus (Drummelsmith *et al.*, 1997; Amor & Whitfield, 1997) and in biosynthetic terms depend on the integral membrane machinery mediated by the Wza (translocation), Wzx (translocation), Wzc (polymerization/translocation) and Wzy (polymerase) proteins (Drummelsmith & Whitfield, 1999). Differences between group 1 and 2 capsules lie in the length of the K_{LPS} chain (Dodgson *et al.*, 1996) and the polysaccharide composition, with polysaccharides of the less diverse group 1 typically containing uronic acids and the more diverse group 2 containing acetamido sugars (reviewed by Whitfield, 2006).

The chemical composition of group 2 (e.g. K1, K5) and 3 (e.g. K10) capsular structures are highly variable but possess several homologous characteristics. Both are expressed in a single form with no link to the LPS lipid A and most have a phosphatidic acid or 3-Deoxy-D-*manno*-oct-2-ulosonic acid (KDO) residue at the reducing terminus of the polysaccharide, thought to mediate attachment to the cell surface (reviewed by Roberts, 1996). However, there are exceptions, notably the PSA-based K1 capsule (detailed in previous sections) which does not appear to interact with the cell surface in this fashion. Instead, it has been proposed that the K1 capsule is anchored to the cell surface by ionic interactions with the negative charges on phosphate groups of the LPS core oligosaccharide (Jiménez *et al.*, 2012). The genes that encode the biosynthetic and export machinery of group 2 and 3 capsules are the *kps* cluster, a relatively well conserved set of genes that are organized into 3 regions. Regions 1 and 3 encode export and translocation proteins, including an ATP-binding-cassette (ABC) transporter (KpsMT), and a serotype-specific set of region 2 genes such as the *neu* cluster of K1-polysaccharide, located near the *serA* chromosomal locus (Silver *et al.*, 1981; reviewed by Whitfield, 2006). There are differences in the organisation of the group 2 and 3 *kps* clusters which account for a major difference between the 2 groups, namely that, in contrast to group 3, group 2 capsule expression is thermoregulated with maximal expression of the capsular genes at 37°C and significantly less expression at lower temperatures (Rowe *et al.*, 2000).

A number of functions have been proposed for capsular polysaccharides. These include prevention of dessication (Ophir & Gutnick 1994) and modulation of biofilm formation (Valle *et al.*, 2006). However, their most well-established function is to provide protection against immune and environmental insults. Although the various K-

antigens do not possess the toxic characteristics of LPS lipid A, they are key virulence factors and contribute to the pathogenicity of *E. coli*. In similar fashion to O-antigens, K-antigen polysaccharides provide resistance to innate host immune processes such as the serum complement cascade and complement–mediated opsinophagocytosis (Howard & Glynn, 1971). Some capsular types provide protection from adaptive immune responses by molecular mimicry of host glycoconjugates; for example, the K1 and K5 group 2 capsular antigens (Troy, 1992; Vann *et al.*, 1981), enable pathogenic strains of *E. coli* to evade immune surveillance mechanisms by capitalizing on the host-age-dependent immune response to TI-2 antigens (Mosier *et al.*, 1977; reviewed by Rijkers *et al.*, 1998).

E. coli strains frequently possess other surface structures of physiological importance. These include peritrichous flagellae (the H-antigens), which are highly complex whip-like structures driven by rotary transmembrane proton-powered motors to provide directional motility (reviewed by Macnab, 1992). Other common structural features are pili (or fimbrae); these are thin protein tubes which protrude from the CM to decorate the bacterial surface where they mediate adhesion to host surfaces (Krogfelt *et al.*, 1990). Although there are a number of pilus types, one specific type serves to illustrate a factor of importance in the natural history of *E. coli*. The F-pilus mediates plasmid-driven conjugation, the transfer of DNA from one cell to another through the tubular F-pilus (reviewed by Ippenihler, 1986), constituting a critical element of horizontal gene transfer (HGT).

Tatum and Lederberg were the first to document the capacity of *E.coli* to directly exchange genetic material (Tatum & Lederberg, 1947), and since this discovery the study of HGT in this species has become a rich area of research. The acquisition of genetic material in HGT can be driven by several mechanisms that include conjugation (transfer of DNA by direct cell-cell contact), transformation (uptake of DNA from the environment) and transduction (introduction of DNA by infection of the bacterium with lysogenic phage; reviewed by Ochman *et al.*, 2000). Codon-bias and G/C base content analysis of the genome of *Escherichia coli* K-12 shortly after publication in 1997 revealed that approximately 18% of chromosomal open reading frames (ORFs) were of foreign origin, with a significant fraction physically associated with mobile genetic elements such as transposon and prophage. It has been estimated that *Escherichia coli* has acquired approximately 16 kb of DNA for each million years since speciation from

its phylogenetic ancestor, *Salmonella enterica*, about 100 million years ago (Lawrence & Ochman, 1998).

Colonization of the GI tract by the majority of *Escherichia coli* strains is commensular in nature, with the GI environmental niche providing the organism with a steady supply of nutrients and a relatively stable, if competitive, environmental medium where *E. coli* may exploit its ability to utilize gluconate as a carbon source more efficiently than other components of the microbiota (Sweeney *et al.*, 1996). The presence of *E. coli* in the GI tract may even provide some mutualistic benefits to the host in terms of resistance to colonization by pathogens (Hudault *et al.*, 2001; Schamberger *et al.*, 2004). However, many strains of *Escherichia coli* are pathogenic, which can be traced to pathogenesis-related determinants, or virulence factors (VFs). These include structures such as the O/K/H antigens and a variety of exotoxins. Many of these VFs can themselves be traced to HGT events which have occurred during the evolutionary history of the microbe. The expanding number of fully sequenced *E. coli* genomes has allowed the comparison of commensals and pathogenic isolates causing different types of infections (also known as different pathovars). It is surprising that the *E. coli* genome has a highly mosaic structure, with only 39% of genes conserved between strains. The large majority of VF genes are either associated with chromosomal pathogenicity islands (PAIs) flanked by mobile genetic elements or associated with plasmids; both provide well-recognised evidence of HGT (Welch *et al.*, 2002). Although the impact on the host of virulence factors, especially those associated with potentially lethal systemic infection, with concomitant dysregulation of the *E. coli* habitat, may at first sight appear to provide little or no benefit in evolutionary terms there must be significant selective pressure in addition to maintenance of microbe-host homeostasis that drives their retention in the *E. coli* population. There is growing evidence that VFs play a significant role in augmenting the ability of *E. coli* to colonize the GI tract (Wold *et al.*, 1992; Nowrouzian *et al.*, 2006) and survive micro-predation (Alsam *et al.*, 2006; Steinberg & Levin, 2007). Both may represent selective pressure for maintainance of beneficial HGT events, with the result that multiple *E. coli* pathovars persist in the environment and continue to cause disease in humans.

1.2.2 One Species, Multiple Pathovars

Although designated as members of a single species, *E. coli* strains are genetically heterogeneous, with pathogenic strains causing infections with mechanistically diverse modes of pathogenesis. Strains that utilize distinct pathogenic mechanisms are grouped together as pathovars; although many elements of pathogenesis are shared, each pathovar has its own unique profile. At present, eight pathovars have been identified and subjected to extensive investigations. They are designated enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC; classified as the separate genus *Shigella*), enteroaggregative (EAEC), diffusely adherent (DAEC), uropathogenic (UPEC) and neonatal meningitic (NMEC). They can be further classified into two groups on the basis of site of infection, namely the intestinal and extra-intestinal pathovars (reviewed by Kaper *et al.*, 2004; Croxen & Finlay, 2010).

1.2.2.1 Intestinal Pathovars

Pathovars which exert their pathogenic effects in the intestine are common mediators of diarrhoeagenic disease in humans and animals as a consequence of disruption of the intestinal epithelium, leading to fluid loss and watery diarrhoea. As noted, the mechanistic basis of disease can vary significantly between pathovars. The diversity in pathogenic mechanisms employed is matched by their diversity with respect to epidemiology, disease associations and mortality in infants, including neonates.

EPEC has an extremely strong association with diarrhoeagenic disease and mortality in neonates and infants younger than 2 months of age in the developing world (reviewed by Levine & Edelman, 1984). EPEC pathogenesis involves the formation of attaching and effacing (A/E) lesions on host intestinal epithelial cells. The bacterium adheres to the cell membrane and induces effacement of the cell microvilli and formation of a pedestal like structure upon which the bacterial cell sits (Moon *et al.*, 1983). The genes involved in A/E lesion formation are clustered in a PAI designated the locus of enterocyte effacement (LEE; McDaniel *et al.*, 1995). The proteins encoded by this locus include the components of a type III secretion system (T3SS) and multiple T3SS-delivered effector proteins. These effectors have a multitude of intracellular

functions. For example, Tir (translocated intimin receptor) protein localizes to the enterocyte apical membrane, binds the bacterial outer membrane protein intimin and promotes close association between the pathogen and host cell (Kenny *et al.*, 1997). Tir subsequently activates host N-WASP and the ARP2/3 complex that mediate the actin cytoskeletal rearrangements which drive A/E lesion and pedestal formation (Kalman *et al.*, 1999). Other effectors include Map, EspF, Nle1 and Cif which inhibit mitochondrial function, disrupt intercellular tight junctions, inhibit solute transport and can induce enterocyte apoptosis (Guttman *et al.*, 2006; Thanabalasuriar *et al.*, 2010; Samba-Louaka *et al.*, 2009).

EHEC is capable of causing severe haemorrhagic diarrhoea in all age-groups. However, subsequent development of potentially fatal haemolytic uremic syndrome (HUS) is significantly more common in young infants, including neonates, and the elderly (Bell *et al.*, 1997). EHEC pathogenesis is similar to EPEC as this pathovar also possesses the LEE PAI and thus forms similar A/E lesions (McDaniel *et al.*, 1995). However, EHEC strains possess additional VF's which mediate greater damage to the intestinal lining and can also cause systemic tissue damage. The characteristic VF of EHEC is the Shiga cytotoxin (Stx; otherwise known as Verotoxin). This multimeric protein binds to Gb3 receptors present on Paneth cells and kidney epithelial cells via its pentameric B subunit (Schuller *et al.*, 2007; Boyd & Lingwood, 1989). This allows intracellular trafficking of the enzymatic A subunit, an *N*-glycosidase which inhibits protein synthesis (reviewed by Nataro & Kaper, 1998). Interestingly, Stx is not secreted by EHEC but is instead released upon lysis of the bacterial cell. This is due to the fact that the cytotoxin is encoded by a lysogenic phage which enters the lytic cycle in response to any DNA damage suffered by its host (Toshima *et al.*, 2007). The intestinal tissue damage mediated by Stx and the other EHEC VF's can result in the systemic dissemination of Stx which can then go on to mediate damage to the kidneys.

The ETEC pathovar is characterized by the production of enterotoxins and has a strong association with mortality in young infants and neonates compared to older children. This may be due to the fact that specific enterotoxin receptors are much more prevalent in the infant intestine compared to adults (Cohen *et al.*, 1988). ETEC enterotoxins are classed as LT (heat-labile) or ST (heat-stable) and any given strain of ETEC may secrete either one or both types. LT's are multimeric proteins with an enzymatic A subunit and a pentameric B subunit which enter host cells via B subunit

binding to the ubiquitous host ganglioside GM1 (Fukuta *et al.*, 1988). Holotoxin internalization and processing releases the A subunit leading to disruption of intracellular cAMP (cyclic adenosine monophosphate) regulation and consequent activation of apical chloride channels. ST's are single peptides which are thought to bind to and activate multiple receptors including guanylatecyclase C (Cohen *et al.*, 1993). ST binding results in increased intracellular cGMP (cyclic guanosine monophosphate) and a similar activation of chloride channels (Forte *et al.*, 1992). Transport of Cl^- into the intestinal lumen results in the osmotic diarrhoea associated with ETEC (reviewed by Sears & Kaper, 1996).

EIEC/*Shigella* strains have a very low infectious dose and infection can be severe in older infants, resulting in fever and inflammatory bloody diarrhoea (dysentery). However, neonatal infection is very rare and characteristically mild (reviewed by Tarlow, 1994). EIEC strains invade the intestinal epithelium by transcytosis of specialized enteric microfold cells (Jensen *et al.*, 1998). The bacterium is phagocytosed by resident macrophages where they induce apoptosis and the release of pro-inflammatory cytokines IL-1β and IL-18. This triggers the inflammatory response that characterizes dysentery (Zychlinsky *et al.*, 1992; Sansonetti *et al.*, 2000). Release from apoptotic macrophage cells allows EIEC to invade the basolateral membranes of enterocytes. This process is facilitated by delivery of intracellular effectors secreted *via* a T3SS. The internalized bacterium subverts cytoskeletal signalling mechanisms and induces the polymerization of F-actin in a uni-directional fashion (Sansonetti *et al.*, 1986). This actin 'tail' propels the pathogen into adjacent enterocytes. The execution of this complex invasive process is mediated by an array of VF's many of which are encoded on the pINV plasmid which encodes the T3SS and 25 secreted effector proteins (reviewed by Schroeder & Hilbi, 2008).

A growing body of evidence indicates that EAEC strains are commonly associated with persistent diarrhoea and are frequently isolated in infants from developing countries, but do not appear to be associated with high mortality rates (reviewed by Nataro & Kaper, 1998). DAEC strains are associated with infections in older infants but, critically, are rare neonatal pathogens and not associated with neonatal mortality (Levine *et al.*, 1993). The pathogenesis of these pathovars is not well understood due to their heterogeneous nature and lack of well-developed animal models to study infection.

Although it is clear that some intestinal pathovars, especially EPEC, ETEC and to a lesser extent EHEC, are associated with diarrhoeagenic disease and mortality in neonates, this should be viewed in the context of the relatively low number of neonatal deaths attributable to diarrhoea, the majority of which are due to rotavirus infection (Tate *et al.*, 2012). The strongest association of *Escherichia coli* with neonatal disease and mortality lies with the extra-intestinal pathovars.

1.2.2.2 Extra-Intestinal Pathovars

The extra-intestinal pathovars of *E. coli* comprise strains which are non-diarrhoeagenic but cause infections in extra-intestinal tissues. It has been proposed that these pathovars should be grouped under the single designation ExPEC (Russo & Johnson, 2000). However, two groups, UPEC and NMEC, are currently recognised on the basis of extra-intestinal tissue tropisms displayed by each disease-causing isolate. Both UPEC and NMEC affect different tissues, are aetiological agents of distinct diseases and utilize distinct repertoires of pathogenic mechanisms.

Colonization of the normally sterile urinary tract and infection of associated tissues are mediated by UPEC strains, which account for approximately 80% of all urinary tract infections (UTIs) in humans (Foxman, 2003). Under normal circumstances, UPEC strains are components of the intestinal microbiota, where they coexist with the host without causing overt symptoms of disease. However the close proximity of rectum and urinary tract may permit transmission from the gut to the genitourinary tract (Russo *et al.*, 1995). UTIs can occur at any age and UPEC strains cause disease in all age-groups, including infants and neonates (Winberg *et al.*, 1973; Foxman, 2003). Even in neonates UTIs are not associated with high mortality rates, although in a small proportion of cases the infection may progress from local to systemic, with the consequence of bacteraemia and sepsis (Biyikli *et al.*, 2004).

Members of the NMEC pathovar may penetrate the CNS of vulnerable neonates to cause meningitis, a potentially lethal inflammatory condition. As previously highlighted, NMEC strains are frequently isolated in such cases. The symptoms of neonatal meningitis and sepsis are essentially identical and strains isolated from cases of neonatal sepsis (termed ExPEC isolates) are generally indistinguishable from *E. coli*

meningitis isolates. As a consequence, non-UPEC ExPEC isolates are grouped within the NMEC pathovar. As the pathophysiology and epidemiology of neonatal bacterial meningitis have been examined previously, the following sections will focus on the molecular epidemiology and pathogenesis of NMEC strains.

1.3 NMEC

1.3.1 The molecular epidemiology of NMEC

Although *E. coli* is a heterogeneous species, with over 170 somatic O-antigens and 80 capsular K-antigens thus far described (reviewed by Raetz & Whitfield, 2002; Whitfield, 2006), the NMEC pathovar is restricted to a very small group of specific serotype combinations. Studies conducted over 35 years ago demonstrated that the most striking and significant element of this group is the K1 capsule. The K1 antigen is a homopolymer of α-2,8-linked *N*-acetyl neuraminic acid (NeuNAc), polysialic acid (PSA), which mimics host PSA (reviewed by Rutishauser, 1996; Troy, 1992); its structure is shown in Figure 1.9. Over 80% of NMEC neonatal meningitis and sepsis isolates express this K-antigen (Robbins *et al.*, 1974; Sarff *et al.*, 1975). Although alternate K-antigens can be found in other NMEC strains, mortality and neurological morbidity rates are significantly higher in K1-expressing isolates (McCracken *et al.*, 1974).

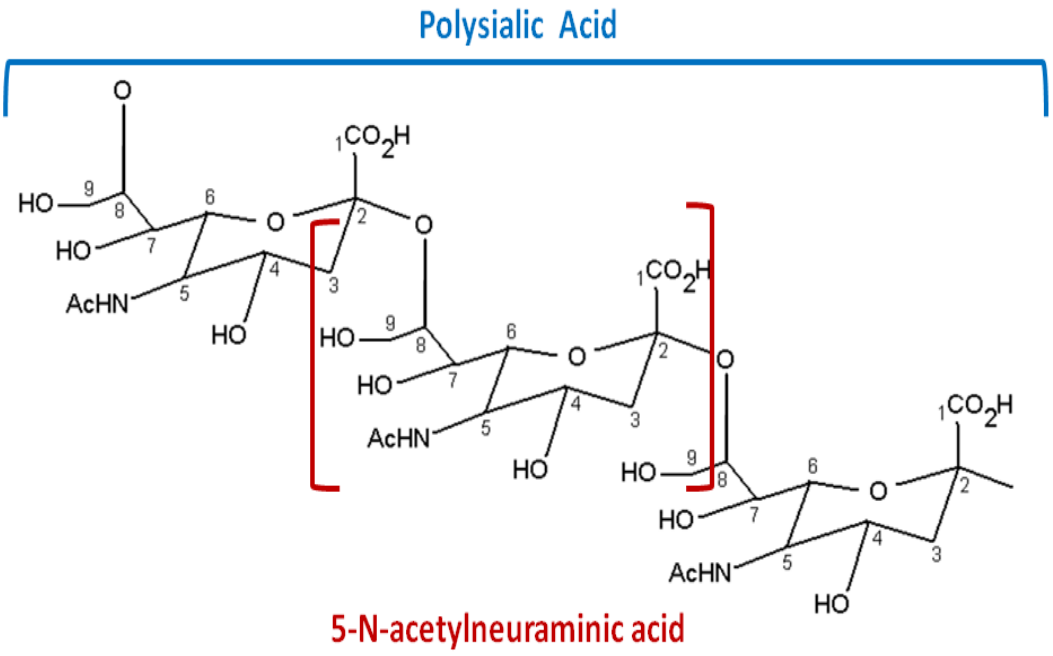


Figure 1.9: *The chemical structure of α-2, 8 linked polysialic acid; one NeuNAc monomer is highlighted.*

NMEC isolates expressing the K1-antigen belong to a restricted number of O- and H-antigen serotypes, with O1, O7, O16 and O18 accounting for almost all NMEC O-serotypes and H6 and H7 flagellar antigens for almost all H-serotypes from human disease isolates (Robbins *et al.*, 1974; Achtman *et al.*, 1983). O1, O7 and O18 NMEC strains are isolated in an approximately equal proportion (~30%) of neonatal sepsis cases; however, O18 serotype strains are found in almost 50% of neonatal meningitis cases and are responsible for a high proportion of lethal events in neonates and experimentally induced animal infections (Pluschke *et al.*, 1983). The serological epidemiology of NMEC indicates that the dominant clonal serotype, with respect to frequency of isolation and severity of disease, is O18:K1:H7, although studies have noted the emergence of virulent O83- and O45-bearing clones in Europe (Bonacorsi *et al.*, 2003; Mulder *et al.*, 1984).

Multi-locus enzyme electrophoresis (MLEE) has been frequently used to phylogenetically differentiate *E. coli* clonal lineages; isolates have been assigned to one of four phylogenetic groups, designated A, B1, B2, and D (Whittam *et al.*, 1983). Analysis of UPEC and NMEC strains has shown that the majority of ExPEC isolates are members of the B2 lineage (Johnson *et al.*, 2001). Further, ribotyping of NMEC strains has revealed that they form a distinct but related sub-group within the B2 lineage, referred to as $B2_1$, indicating that the NMEC pathovar and other ExPEC strains are descended from a common ancestor which acquired the VFs necessary for survival and pathogensis in the extra-intestinal environment (Bonacorsi *et al.*, 2003).

The K- and O-antigens are major VFs of NMEC; however, other genetic loci are also common to many NMEC isolates and are frequently associated with highly virulent serotypes, such as O18:K1:H7 strains. These include loci encoding the type-1, P- and S-pili (*fimH, papG, sfa*), α-haemolysin (*hylA*), cytotoxic necrotizing factor 1 (*cnf1*), Hek adhesin/invasin protein (*hek*), Ibe invasin proteins (*ibeA/B/C*), TraJ protein (*traJ*), OM protein A (*ompA*), arylsulfatase-like A (*aslA*) and the siderophore receptors for salmochelin, yersiniabactin and aerobactin (*iroN, fyuA, iucA/C*), as well as uncharacterized NMEC-specific PAIs (Moulin-Schouleur *et al.*, 2006; Watt *et al.*, 2003; reviewed by Xie *et al.*, 2004; Bonacorsi & Bingen, 2005). In many NMEC strains, the siderophores and other VFs can be localized to a large 134 kb mobile plasmid, pS88,

which plays a role in virulence, in particular survival in the host's blood compartment (Peigne *et al.*, 2009).

Based on serology, phylogenetics and distribution of VFs in NMEC isolates, the molecular epidemiology of NMEC strains clearly indicates that meningitis and sepsis isolates of *E. coli* belong to a closely related sub-group of the species. These pathogens have usually been identified from the presence of their most conserved and possibly most important VF, the K1 capsule, and are designated *E. coli* K1.

1.3.2 Pathogenesis of *E. coli* K1 infection

The pathogenesis of *E. coli* K1, from gastrointestinal colonization to penetration of the CNS, is a multi-step process involving attachment to, and invasion of, multiple host cell types, transcytosis across two formidable biological barriers and survival in an extremely hostile environment. Although the major *E. coli* K1 VF, the capsular polysaccharide, plays an indispensible role in neonatal pathogenesis, an array of other VFs are implicated in the translocation of the bacterium from the intestinal lumen to the CSF. The major steps towards CSF penetration and disease causation, as well as the roles of the bacterial factors involved, are summarized in Figure 1.10.

To cause systemic infection, pathogens must first gain access to the blood compartment. In the absence of direct entry due to a breach in the skin-barrier, this necessitates colonization of host mucosal surfaces. With *E. coli* K1, this is generally presumed to be the colon and distal small intestine (Sarff *et al.*, 1975; Pluschke *et al.*, 1983). This is a reasonable assumption given that these sites are heavily colonized by the pathogen in animal models (Glode *et al.*, 1977; Plushke *et al.*, 1983) and the bacteria are well-adapted to niches within the GI tract. Genetic analysis of *E. coli* K1 mutants unable to colonize the GI tract has revealed several proteins which are vital to the capacity of the pathogen to survive in this niche (Martindale *et al.*, 2000). These include the type-1 pilus adhesin FimH, proteins involved in adaptation to anaerobic respiration and a bile salt efflux pump; all are clearly necessary for survival in the GI tract. Interestingly, this work highlighted the importance of four proteins of unknown function, termed DgcA-D. All have homologues in strains belonging to other *E. coli* pathovars, with the exception of DgcD which appears unique to *E. coli* K1.

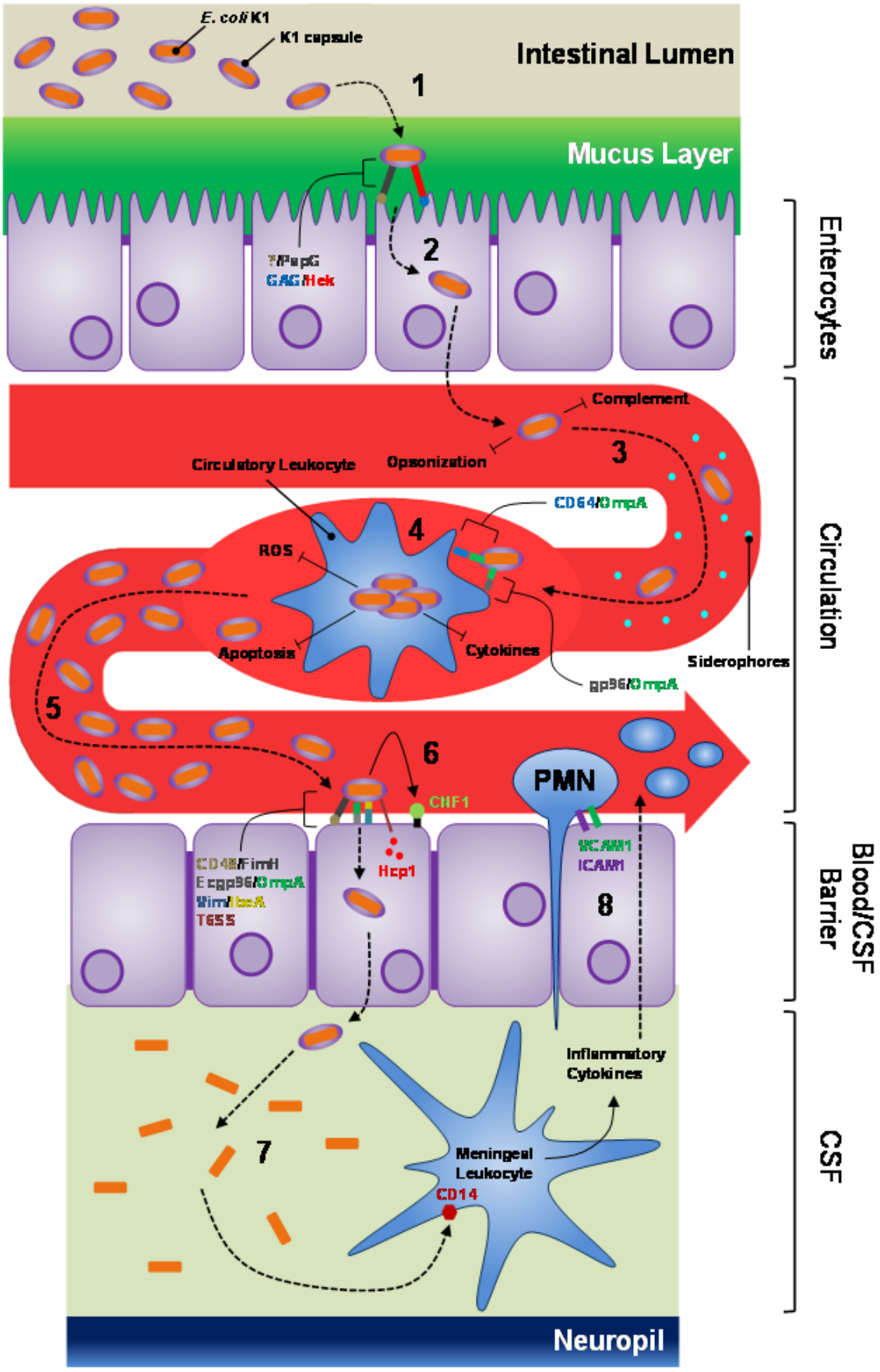

E. coli K1
K1 capsule
Intestinal Lumen
1
Mucus Layer
?/PapG
GAG/Hek
2
Enterocytes
Complement
Opsonization
3
Circulatory Leukocyte
CD64/OmpA
4
ROS
Apoptosis
Cytokines
gp96/OmpA
Siderophores
Circulation
5
6
CNF1
PMN
CD48/FimH
Ecgp96/OmpA
VimI/IbeA
T6SS
Hcp1
VCAM1
ICAM1
8
Blood/CSF Barrier
Inflammatory Cytokines
7
Meningeal Leukocyte
CD14
CSF
Neuropil

Figure 1.10: *The pathogenesis of neonatal E. coli K1 infection and induction of meningitis is a multi-step, multifactorial process.* ***1****; E. coli K1 colonizes the intestines and penetrates the intestinal mucous layer.* ***2****; the bacterium adheres to and subsequently invades enterocytes of the intestinal epithelium, a process mediated by the binding of bacterial Hek to enterocyte glycosaminoglycan (GAG) and possibly involving the PapG pilus adhesin.* ***3****; transcytosed bacteria penetrate the blood compartment where the K1 capsule inhibits complement deposition and innate/adaptive opsonisation and secreted siderophores scavenge free iron.* ***4****; the bacterium invades circulating leukocytes by binding to the CD64 receptor on macrophages or gp96 on neutrophils through outer membrane protein A (OmpA), which also promotes intracellular survival and replication by inhibition of leukocyte apoptosis and the release of cytokines and reactive oxygen species (ROS).* ***5****; circulating bacteria replicate to a CNS-invasion threshold level of* $>10^3$ *CFU/mL of blood.* ***6****; bacterial adherence to and invasion of endothelial cells of the blood/CSF barrier is mediated by binding of FimH, OmpA and IbeA bacterial surface proteins to cellular CD48, Ecgp96 and vimentin (Vim) respectively. Secreted cytotoxic necrotizing factor 1 (CNF1) and Hcp1 effector secreted by a type VI secretion system (T6SS) are also involved in the invasive process.* ***7****; bacteria transcytose into the cerebrospinal fluid (CSF) where the K1 capsule is no longer expressed by the pathogen, exposing immunogenic LPS. The lipid A moiety of LPS is detected by CD14 receptor of meningeal leukocytes resulting in secretion of pro-inflammatory cytokines.* ***8****; Cytokine secretion stimulates expression of adhesion molecules ICAM1 and VCAM1 on endothelial cells, which mediate extravasation of polymorphonuclear leukocytes (PMN) into the CSF, resulting in meningeal inflammation. Host receptors binding bacterial ligands are displayed in receptor/ligand colour-coded format.*

Stable colonization is the first step in the pathogenic process, followed by adhesion, to, and invasion of, GI epithelial enterocytes, mediated in part by the P-pilus adhesin PapG and the Hek protein. The role of pilus-based adhesins in adhesion to the GI epithelium is unsurprising when one considers their role in binding urinary tract epithelia in UPEC strains (Bahrani-Mougeot *et al.*, 2002; Korhonen *et al.*, 1986). However the precise role of each pilus type in *E. coli* K1 enterocyte adherence is less clear. Although type-1 pili are essential for colonization, they do not appear to play a

significant role in enterocyte adhesion, whereas the P-pilus, which was originally associated with kidney cell adherence, does appear to bind enterocyte membranes and therefore may play a role in the adhesion of *E. coli* K1 (Tullus *et al.*, 1992; Wold *et al.*, 1992; Goetz *et al.*, 1999). Studies in bladder epithelial cells and polarized enterocyte epithelial cell lines have revealed that *E. coli* K1 can invade and transcytose these cells *in vitro* (Burns *et al.*, 2001) and that invasion involves manipulation of the enterocyte cytoskeleton (Meier *et al.*, 1996). The only factor directly implicated in epithelial cell invasion is the Hek protein, an OM protein which confers an invasive phenotype in recombinant non-invasive *E. coli* strains (Fagan & Smith, 2007). This protein has a putative β-barrel structure with adhesion and invasion dependent on a 25-amino-acid loop which mediates binding to the glycosaminoglycan moieties of host cell surface proteoglycans (Fagan *et al.*, 2008). As yet, however, the invasive mechanism mediated by Hek is unclear and its relevance to pathogenesis has not been confirmed *in vivo*.

If *E. coli* K1 traverses the intestinal epithelium, how does it gain access to the epithelial cells? The intestinal epithelium is coated by a layer of gel-forming mucins (for example, Muc2 in the colon), which forms a bilayered structure, with the inner stratified mucin layer functioning as an exclusion barrier, physically separating the bacteria of the intestinal microbiota from the enterocyte cell surface (Johansson *et al.*, 2008). Although pathogenic bacteria can penetrate this layer (Bergstrom *et al.*, 2010), the enabling processes have not been characterized in any intestinal *E. coli* pathovars. This lack of understanding, coupled with incomplete knowledge of adhesion and invasion mechanisms permitting transcytosis of the intestinal epithelium *in vivo*, illustrates that *E. coli* K1 translocation of the intestinal mucosa is the least well characterized step in the pathogenic process.

The survival and replication of the pathogen in the vascular compartment is comparatively well characterized. The K1 capsular antigen is the critical determinant of the capacity of *E. coli* K1 to survive in the bloodstream (Kim *et al.*, 1992), in part due to the molecular mimicry of endogenous host PSA. K1-antigen inhibits adaptive immune responses to the bacterium by inhibition of immunoglobulin-mediated opsonisation and its capacity to contribute to serum resistance through inhibitory modulation of the alternative complement pathway (Leying *et al.*, 1990; Mushtaq *et al.*, 2004). The O-antigen polysaccharide acts synergistically with the K-antigen with respect to complement inhibition by disrupting activation of the classical pathway (Burns & Hull,

1998). Interference with complement activation is also mediated by OmpA which sequesters C4b-binding protein (C4bp), an inhibitory component of the complement cascade which promotes the degradation of C4b and C3b, essential components of the activated complement cascade (Wooster *et al.*, 2006).

The availability of free iron is a factor limiting bacterial growth and survival *in vivo*. Bacteria generally require a cytoplasmic concentration in the 10^{-5}-10^{-7} M range (reviewed by Andrews, 2003) but only 10^{-24} M is present in human serum (reviewed by Fischbach *et al.*, 2006). Bacteria secrete iron-chelating siderophores which compete for free iron and are recognized by high affinity receptors at the bacterial surface to facilitate transport back into the cell. *E. coli* K1 strains produce a range of siderophores and associated receptors; however, bloodstream survival is dependent on the siderophore salmochelin and its receptor IroN (Nègre *et al.*, 2004; Peigne *et al.*, 2009).

Factors other than resistance to complement and iron acquisition contribute towards the capacity of *E. coli* K1 to survive in the blood compartment. The bacteria are able to invade circulating leukocytes and replicate within them, utilizing these key immune cells as a reservoir for systemic growth. *E. coli* K1 binds to and invades macrophages in an opsonisation-independent manner (Sukumaran *et al.*, 2003). This process is mediated by binding of bacterial OmpA to the α-chain of the macrophage receptor CD64 (Fcγ receptor Ia). Uptake of the bacterium is effected by manipulation of the macrophage cytoskeleton and the bacterium is sequestered in a vacuole within the cytoplasm, where it is able to replicate and avoid phagosome-lysosome fusion. Depletion of the macrophage population renders neonatal mice resistant to systemic *E. coli* K1 infection (Mittal *et al.*, 2010). Internalized *E. coli* K1 not only replicate in the macrophage but also ensure preservation of this replicative niche by activating the host anti-apoptotic mediator Bcl_{XL} (Sukumaran *et al.*, 2004). Furthermore intracellular *E. coli* K1 inhibits the phosphorylation and degradation of IκB, the negative regulator of NFκB activation, thus preventing the production of pro-inflammatory cytokines; Selvaraj *et al.*, 2005).

E. coli K1 is able to utilize neutrophils in a similar fashion (Mittal *et al.*, 2011). Again, OmpA is the critical determinant of this process through its capacity to bind to gp96 neutrophil receptors prior to internalization. Neutrophil-internalized *E. coli* K1 reduces the expression of NADPH oxidase complex proteins, preventing the generation

of reactive oxygen species (ROS) and crippling the oxidative burst process used by the neutrophil to degrade phagocytosed bacteria. Interestingly, this study also demonstrated that depletion of the neutrophil population rendered neonatal mice resistant to systemic *E. coli* K1 infection, indicating that replication in both macrophages and neutrophils is critical to *E. coli* K1 survival in the bloodstream. Initially, neutrophils are colonized; macrophages subsequently also provide a replicative niche. This sequence may reflect the relatively short lifespan of neutrophils in comparison to the more long-lived macrophage population.

Intracellular replication in circulating leukocytes and *E. coli* K1's significant resistance to serum-mediated clearance results in the bacterial load of the pathogen increasing in the host's bloodstream until a critical threshold, empirically determined to be approximately 10^3 CFU/mL, is reached which precipitates invasion of the CNS (Dietzman *et al.*, 1974). This requires transversal of an endothelial barrier which has evolved, even more so than the gastrointestinal barrier, to isolate the tissues which it protects; the blood-brain barrier (BBB).

Although a significant proportion of *E. coli* K1 research has been dedicated to the biomechanics of CNS invasion, the site of translocation into the CNS remains controversial. The BBB comprises two interfaces between the CNS and the vasculature. The larger interface is formed by the microvascular endothelial cells of the capillaries which penetrate the CNS, henceforth referred to as the endothelial barrier. The smaller is the blood-CSF barrier (BCSFB) formed by the fenestrated endothelium of the capillaries surrounded by epithelial cells of the choroid plexus (reviewed by Abbott *et al.*, 2010). Whilst some research has indicated that *E. coli* K1 is associated with the endothelial barrier and not the BCSFB (Kim *et al.*, 1992), others have indicated that the BCSFB is the more likely site of pathogen-CNS association (Parkkinen *et al.*, 1988; Zelmer *et al.*, 2008). Evidence obtained from investigations of the capacity of other pathogens to access the CNS indicates that both translocation sites can be exploited by neuropathogens. Thus, the site of translocation varies between species, with the BCSFB implicated in *H. influenzae*, *N. meningitidis* (which can also transverse the endothelial barrier) and *Streptococcus suis* infections (Daum *et al.*, 1978; Pron *et al.*, 1997; Tenenbaum *et al.*, 2009) and the endothelial barrier in *S. pneumoniae* infections (Zwijnenburg *et al.*, 2001; Fillon *et al.*, 2006). The capacity to cross the endothelial barrier implies a pathogen must access the neuropil (the neuron-containing brain

parenchyma), as the capillaries of the cerebral vasculature form a network throughout the brain and, in contrast to the postcapillary venules from which the capillaries branch, are not surrounded by a perivascular space containing CSF (reviewed by Bechmann *et al.*, 2007). *E. coli* K1 has been observed in the perivascular space and it was therefore proposed that translocation occurred at the endothelial barrier (Kim *et al.*, 1992). However, *E.coli* K1 has not been observed in the neuropil (Kim *et al.*, 1992; Zelmer *et al.*, 2008). This contrasts with invasion by *S. pneumoniae*, which is widely distributed in brain tissue (Fillon *et al.*, 2006). This represents compelling evidence that the pathogen utilizes the BCSFB as the site of entry to the CNS, rather than the endothelial barrier.

The vascular endothelial barrier and the epithelial BCSFB are comprised of cells that are intimately connected by intercellular tight junctions and adherens junctions (reviewed by Abbott *et al.*, 2010). These junctions provide the barrier function of the BBB interfaces, severely inhibiting the paracellular movement of molecules. Invading pathogens must either disrupt these junctions or utilize the transcellular pathway in order to gain access to the CNS. *E. coli* K1 is believed to use the transcellular route to migrate across this barrier and a significant volume of research has focused on the mechanics of this process. The bulk of this work has focused on *in vitro* interactions of human brain microvascular endothelial cells (BMEC) and *E. coli* K1 and may not be representative of interactions *in vivo* if the pathogen does not invade the CNS through the vascular endothelium. However this does not mean that the factors and mechanisms identified by this work are irrelevant, as *in vitro* studies have been complemented *in vivo* using single locus isogenic *E. coli* K1 mutants that indicate their importance to the penetration process.

Adhesion to BMEC cells is mediated by multiple factors, some of which are involved in intracellular invasion. One is the FimH adhesin of the type-1 pilus, which binds mannosylated glycoconjugate receptor CD48 on the cell surface (Khan *et al.*, 2007). OmpA is involved in adhesion through binding to Ecgp96, a homologue of the gp96 receptor employed by the bacterium to adhere to neutrophils (Pascal *et al.*, 2010). S-pilus adhesin (Sfa) binding to sialoglycoprotein receptors has not been considered critical for BMEC adhesion, although it does occur (Prasadarao *et al.*, 1997). However, this view does not take into account the fact that S-pili have a much stronger affinity for the choroid epithelial cells of the BCSFB than for BMEC cells (Parkkinen *et al.*, 1988).

The invasion and transcytosis of endothelial cells is a multifactorial process. CNF1, a Rho GTPase activating secreted bacterial toxin (reviewed by Lemonnier *et al.*, 2007) contributes to *E. coli* K1 invasion in similar fashion to its role in UPEC (Khan *et al.*, 2003). The toxin binds cellular the 37 kDa laminin receptor precursor (37LRP) on the endothelial cell surface, activating RhoA and mediating actin filament formation at the site of bacterial entry (Khan *et al.*, 2002). FimH binding also triggers RhoA activation (Khan *et al.*, 2007). This mechanism is complemented by OmpA which, after binding to Ecgp96, activates cellular PI3K (phosphatidylinositol 3-kinase), resulting in actin condensation (Prasadarao *et al.*, 1999; Khan *et al.*, 2003). Another critical factor in *E. coli* K1 invasion is IbeA, which initially binds to the receptor vimentin, an intermediate filament protein of the cellular cytoskeleton (Zou *et al.*, 2006). IbeA and OmpA binding to their cognate receptors induce the activation of STAT3 (signal transducer and activator of transcription 3) which activates the Rho GTPase Rac1, mediating further cytoskeletal rearrangements (Maruvada & Kim, 2012). A recent addition to the mechanisms utilized by the pathogen to invade BMEC cells is a type VI secretion system (T6SS). These complexes are thought to deliver effector proteins to host cells by a mechanism that mimics the tail spike of the T4 bacteriophage (Pukatzki *et al.*, 2009). The T6SS-secreted effector Hcp1 has been implicated in interactions leading to *E. coli* K1 invasion (Zhou *et al.*, 2012). The cumulative actions of these invasion factors lead to the internalization of *E. coli* K1 within a vacuole. The pathogen does not replicate within the vacuole but survives transit through the cell; survival is dependent on the presence of the K1-capsule (Hoffman *et al.*, 1999). Intracellular vacuoles containing K1-encapsulated *E. coli* are not targeted for lysosomal fusion by cellular endosomal maturation mechanisms; the role of the capsule in this process is presently unclear (Kim *et al.*, 2003).

Transcytosis of the BBB allows access of *E. coli* K1 to the CSF, where bacterial cell division invariably takes place. As described earlier, bacterial growth in the CSF stimulates the production of pro-inflammatory mediators by CNS leukocytes, leading to infiltration of polymorphonuclear leukocytes (PMNs) as part of an inflammatory response that is the primary mediator of cerebral damage associated with meningitis. Massively increased expression and production of chemokines and other cytokine inflammatory mediators have been documented in experimental *E. coli* K1 infection (Zelmer *et al.*, 2010). However, there is another intriguing aspect to this final step in *E.*

coli K1 pathogenesis. Once the bacterium has penetrated the CSF and colonized the meninges, there appears to be a marked reduction in detectable K1-antigen at the surface of the bacterial cell (Zelmer *et al.*, 2008). Removal of the protective capsule in this environment has clear implications with respect to the inflammatory response within the CNS, as it would expose highly immunogenic LPS to resident leukocytes, prompting a rapid inflammatory reaction mediated by LBP/CD14 interactions with TLR4, facilitating opsonisation and phagocytosis of the pathogen by leukocytes. Such rapid-onset inflammatory events induced by exposure to pro-inflammatory mediators may contribute to the severe mortality and morbidity of *E. coli* K1 meningitis (Stoll *et al.*, 2002ab; Harvey *et al.*, 1999). The mechanistic basis of capsule reduction has not yet been determined. It is possible that exposure to host factors in the CNS induces removal of the capsular structure; this could involve the recently-identified sialidase Neu4, which hydrolyzes α2, 8 linked PSA and is used by the host to regulate NCAM adhesion (Takahashi *et al.*, 2012).

Thus, the pathogenic processes associated with neuroinvasive *E. coli* K1 form a complex multi-step process that has evolved to circumvent an array of host mechanisms. The pathogen has the capacity to survive and subvert these mechanisms using a palette of VFs, which together allow the bacterium to colonize the inhospitable environment of the GI tract and cause potentially fatal disease in neonates. However, in this regard a key pathogenesis-related question remains unanswered; in light of the clear pathogenic potential of this microorganism, why is *E. coli* K1-mediated disease prevalent in the neonatal population but not in adults? An answer to this question would provide a clearer understanding of *E. coli* K1-mediated neonatal disease and may provide clues as to how to prevent it.

1.4 The age-dependency of *E. coli* K1 infection

1.4.1 The basis of age-dependency

Sepsis and meningitis due to *E. coli* K1 is strongly dependent on the age of the host. The pathogen is isolated in over 80% of cases of neonatal meningitis where *E. coli* is determined to be the aetiological agent (Robbins *et al.*, 1974; Sarff *et al.*, 1975); the organism only very rarely causes systemic infection in older infants and adults (Pitt, 1978; Sarff *et al.*, 1975). Age dependency is especially interesting in the context of *E. coli* K1 carriage in different age groups of the general population, as shown by Sarff and colleagues in 1975 (Figure 1.11).

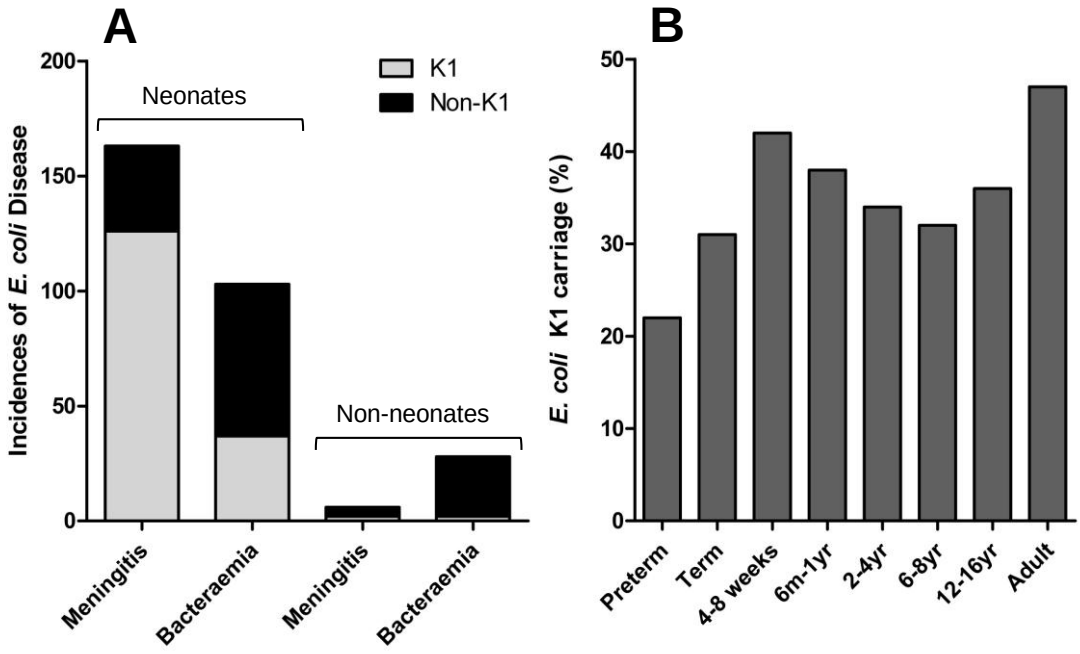


Figure 1.11: *(A). Proportion of E. coli meningitis and bacteraemia isolates expressing K1 antigen in neonatal and non-neonatal infections; (B) rate of carriage of E. coli K1 in different age-groups, as determined by rectal swab culture. Data from Sarff et al., 1975.*

The rate of carriage does not positively correlate with incidence of *E. coli* K1 disease, the 'at risk' population (the neonates) displays a lower incidence of carriage than older infants and adults, both of whom have higher overall rates of carriage but a much lower incidence of disease. This data indicates that, although *E. coli* K1 acts as a pathogen in the neonatal population, it has a commensular lifestyle in older infants and adults. The age dependency of systemic infection has been confirmed in rodent models of *E. coli* K1 infection, showing that age dependency is not restricted to human infections (Glode *et al.*, 1977; Bortolussi *et al.*, 1978; Pluschke & Pelkonen, 1988). This raises the question as to which factors influence this change from susceptibility to resistance to infection and at what stage in the pathogenic process of *E. coli* K1 disease do they act? In other words, which elements of the host mediate the development of resistance to systemic infection?

Few studies have addressed these important questions and none provide definitive evidence for specific resistance mechanisms, but they do provide some indication as to which host factors are not involved in the determination of age dependency. For example, it has been shown that type 1- and S-pilus-mediated adhesion of *E. coli* K1 to host cells is an age-independent process (Schroten *et al.*, 1992; Clegg *et al.*, 1984). The capacity of the pathogen to invade BMEC cells harvested from humans and animals of different age groups is also age independent (Stins *et al.*, 1999). *E. coli* K1 survival in the blood circulation and penetration of the CNS after systemic administration to animals of differing ages has also been examined. Although a higher dose of *E. coli* K1 is required to induce meningitis in older animals (not unexpected given the size differences between neonatal and adult rodents), the pathogen survives in the adult bloodstream and penetrates the CNS (Bortolussi *et al.*, 1978; Pelkonen & Pluschke, 1989; Kim *et al.*, 1992). Studies of GI tract colonization by *E. coli* K1 in relation to susceptibility to systemic infection suggest that age dependency may be determined, at least in part, by the capacity of the bacterium to translocate from the gut to the blood circulation. Although *E. coli* K1 may colonize the GI tract of rats at any age, younger neonates were found to be susceptible to systemic infection following colonization of the GI tract, whereas older neonatal rats were not, although colonization rates were lower in the older cohort (Glode *et al.*, 1977). In a more recent study, Mushtaq and colleagues also demonstrated lower rates of bacteraemia in older neonatal rats despite comparable rates of intestinal colonization (Mushtaq *et al.*, 2005). These

studies demonstrate a very close correlation between bacteraemia and mortality, suggesting that systemic dissemination is not age related once the bacteria have entered the bloodstream, again implicating penetration of the intestinal barrier as the source of variability. Despite these observations, the relationship between the age of the infected individual and intestinal translocation of *E. coli* K1 has not been interrogated. In considering this issue, the host tissue and the microbial population which comprises the intestinal microbiota must be taken into account.

1.4.2 The intestinal microbiota

With an estimated 4-6 nonillion (10^{30}) prokaryotic cells comprising 350-550 billion metric tonnes of carbon and representing tens of billions of genes, the bacterial superkingdom outstrips all other forms of life on the Earth in terms of biomass and biodiversity, as well as in importance to the global biosphere (reviewed by Whitman *et al.*, 1998). A small proportion of this vast global micro-ecology inhabits the external environ and internal mucosal surfaces of multi-cellular organisms of the animal kingdom. Mammals, including humans, are densely colonised by microorganisms. Diverse, yet specialised communities of organisms inhabit the skin, urogenital tract, nasal and oral cavities and GI tract, with the number of bacteria estimated to be between ten and one hundred times greater than the combined total of somatic and germ cells of the colonized host (reviewed by Berg, 1996).

Of all the colonized regions of the mammalian organism, the GI tract, in particular the large intestine that comprises the various colonic elements, is most heavily populated by microorganisms. The large majority of these are bacteria and a small proportion belong to the Archaea and Eukarya (Eckburg *et al.*, 2003). The typical adult human intestine contains 100 trillion microbes (10^{14}), with 10^{11}-10^{12} microbes per millilitre of colonic luminal content (Ley *et al.*, 2006). At the species and genus levels, this population varies significantly between individuals; however, metagenomic analyses have shown the phyla Firmicutes, Bacteriodetes and Proteobacteria are the dominant organisms within this niche (Gill *et al.*, 2006; Palmer *et al.*, 2007). The GI tract microbiota possesses in excess of 100 times as many genes as the mammalian

nuclear genome (Gill *et al.*, 2006), constituting a microbiome that has co-evolved with the human genome and which impacts significantly on human metabolism and health.

For example, the capacity of the gut to absorb fibrous components and long chain polysaccharides such as cellulose is due to prior digestion by the microbiome (Flint *et al.*, 2007). Members of the microbiota have been implicated in the regulation of host fat storage (Bäckhed *et al.*, 2004). The gut microbiota is intimately involved in vitamin biosynthesis and lipid and mineral metabolism (reviewed by Resta, 2009). These and other bacterial influences on the gastrointestinal contents provide the mammalian gut with enhanced metabolism in terms of both efficiency and capability.

The microbiota plays a key role in the orderly development of gut tissues and the immune response and in protection of the host from enteric disease. It must also concomitantly compete with opportunistic and obligate pathogens for resources. It plays an important role in the regulation of angiogenesis (Stappenbeck *et al.*, 2002) and the development of humoral and cellular mucosal immune processes through interactions with gut-associated lymphoid tissues (GALT; reviewed by Cebra, 1999; Round & Mazmanian, 2009). Intestinal colonization by bacterial species such as *Bacteroides thetaiotaomicron* and the segmented filamentous bacteria induce the gut to secrete antimicrobial peptides (AMPs) such as angiogenins and REG3γ (Keilbaugh *et al.*, 2005) In terms of pathogen-protection, probiotic organisms such as the lactobacilli stimulate mucin production by intestinal epithelial cells, compromising adhesion of pathogenic *E. coli* (Mack *et al.*, 1999). This protective mechanism is part of a group of related processes termed colonization-resistance, affording protection of the host from opportunistic and obligate pathogens by the competitive dynamics of the endogenous commensal/mutualistic bacterial population, considered a primary beneficial function of the microbiota (Endt *et al.*, 2010; reviewed by Vollaard & Clasener, 1994). The influence of the microbiota on health is, however, not solely beneficial. Specific groups of organisms have been implicated in the development of gastric cancer (Blaser *et al.*, 1995), inflammatory bowel disease (Ott *et al.*, 2004) and NEC (Hoy *et al.*, 2000; De La Cochetière, 2004).

The temporal development of the human intestinal microbiota varies between individuals; however, general trends are evident due to the application of metagenomic techniques and DNA sequencing technology (Figure 1.12). It has long been thought that

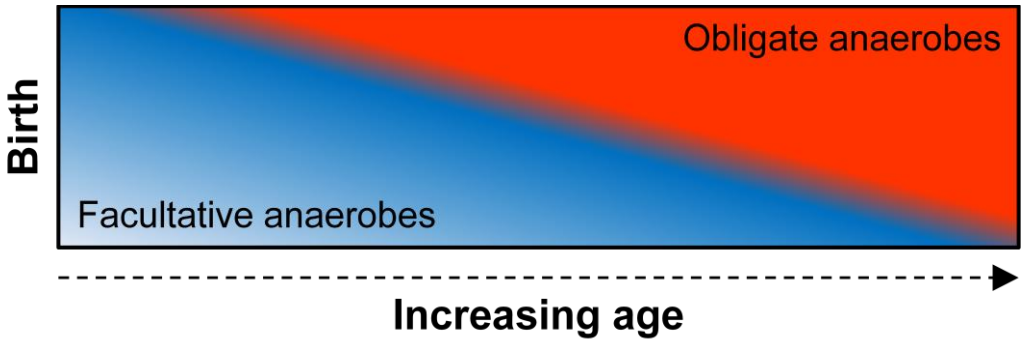


Figure 1.12: *Changes in the relative proportions of facultative (blue) and obligate (orange) anaerobes in the neonatal intestinal microbiota.*

during the gestation period in the absence of *in utero* complications, the foetal gastrointestinal tract remained sterile. *Post-partum*, the neonate is rapidly colonised by microorganisms in successive waves which, over time, cumulate into a climax community representing an adult-like microbiota (Favier *et al.*, 2002). The early colonisation period tends to be dominated by single taxonomic groups, usually facultative anaerobes such as *Enterobacteriaceae*, *Streptococcus* and *Staphylococcus* spp. These reduce the oxygen tension within the intestines and facilitate later colonisation and domination by obligate anaerobes such as Eubacteria and Clostridia (Palmer *et al.*, 2007). Acquisition of these colonizers is dependent on environmental factors and vertical transmission from the cutaneous, vaginal and colonic maternal microbiota (Bettelheim *et al.*, 1974, Schwiertz *et al.*, 2003).

The GI tract is a complex environment comprising microbe-host interactions in conjunction with interactions between members of the microbiota. This system is finely balanced and dependent on a multitude of factors. In the neonate, the microbiota is a dynamic entity, with significant micro-ecological shifts as the host develops. These alterations may increase the colonization-resistance of the intestine and impact on the capacity of *E. coli* K1 to access and translocate across the enterocyte epithelium. Interactions between members of the microbiota inhibit the adhesion and toxin secretion of EHEC strains (Mack *et al.*, 1999; de Sablet *et al.*, 2009) and compromise the pathogenesis of other enteric pathogens (Pultz *et al.*, 2005; Endt *et al.*, 2010). In addition, the microbiota stimulate development of the GALT (reviewed by Cebra, 1999;

Round & Mazmanian, 2009); such maturation of host tissues may ensure that the GI tract becomes refractory to bacterial translocation across the intestinal epithelium.

1.4.3 The intestinal tissues

The internal surface area of the adult human intestinal tissues is approximately two million cm^2. The GI tract undergoes significant morphological and cytological differentiation in the postnatal period. This period comprises Phase IV in the ontogeny of mammalian intestinal development and is defined by the changes that occur in response to stimulation through exposure to enteral nutrition (i.e. maternal milk) and a glut of novel antigenic material to which the neonate must develop an effective tolerance in order to survive in the extra-uterine environment (reviewed by Wagner *et al.*, 2008). The developmental process is highly complex and controlled by a swathe of highly conserved genes, such as the hedgehog, Notch, SOX, and WNT pathway mediators (reviewed by Barbara *et al.*, 2003). Despite its complexity there are several key features of the development process which may be relevant in the context of susceptibility to *E. coli* K1 colonization and infection.

The neonatal proteome is altered in age dependent fashion during postnatal development of the intestine (Hansson *et al.*, 2011). Changes in the biochemical physiology of the tissues affect their digestive and absorptive properties as they mature towards the adult phenotype (reviewed by Henning, 1979). It has been established that the neonatal intestine is permeable to macromolecules such as intact proteins and sugars (Weaver *et al.*, 1984) due to macropinocytosis, a form of endocytosis similar to phagocytosis (reviewed by Swanson & Watts, 1995). The neonatal intestine utilizes this process to acquire macromolecules prior to the development of a more mature digestive capacity; it also mediates the maternal-neonatal transfer of passive immunity by absorption of secretory IgA molecules present in breast milk (reviewed by Wagner *et al.*, 2008). Cessation of macromolecular uptake, or gut closure, occurs at different times *post-partum* in different mammalian species (Lecce *et al.*, 1973). In humans, it may occur as early as three days *post-partum* (Vukavic, 1984). It is possible to speculate that the increased permeability of the neonatal intestine plays a role in *E. coli* K1 epithelial

translocation, although this would be dependent on the time of gut closure and its correlation with the epidemiology of *E. coli* K1 infection.

At the anatomical level the intestines are fully formed *pre-partum* but at the cellular level a significant degree of differentiation occurs *post-partum*, a process which is driven by the intestinal epithelial cells. The intestinal epithelium of the late foetus already possesses long protruding villous structures in the small intestine, but the perinatal epithelial development of both large and small intestinal compartments is characterized by the formation of tubular invaginations, or crypts. At the base of crypts lie the pluripotent stem cells, the motors of cellular differentiation, and from which the various cellular subpopulations of the intestine arise (reviewed by van der Flier & Clevers, 2009; Barbara *et al.*, 2003). Intestinal cellular differentiation gives rise to four primary intestinal cell lineages. The most prevalent is the enterocyte, which constitutes ~80% of epithelial cells in the adult intestine. These are the absorptive workhorses of the intestine and are highly polarized, with apical membrane microvilli serving to massively increase the absorptive surface area of the gut. The second is the enteroendocrine cell, which account for only 1% of the intestinal epithelium yet comprises the largest population of hormone-secreting cells in the body and has a critical regulatory role in intestinal function (reviewed by Schonhoff *et al.*, 2004). The remaining two cellular lineages are goblet cells and Paneth cells, both of which contribute in different ways to the defence of the intestine; thus, the developmental regulation of their differentiation may impact on *E. coli* K1 infection.

Paneth cells, named after the Austrian physician Joseph Paneth who first described them in 1888, are highly granulated cells with densely packed rough endoplasmic reticuli that are spatially restricted to the bottom of the small intestinal crypts. Unlike other enteric epithelial lineages, they do not migrate along the villi as they mature. In humans, there are usually between 5-15 cells per crypt; they are present in most, but not all, mammalian species. Although they may play a role in digestion and regulation of crypt development, their primary function is the production and secretion of a range of antimicrobial peptides (AMPs) which modulate the microbiota and maintain intestinal homeostasis (reviewed by Porter *et al.*, 2002; Bevins & Salzman, 2011). Paneth cells produce a range of AMPs, including the constitutively expressed α-defensins, defensin-related peptides, lysozyme C, phospholipase A2 and the inducible REG3 C-type lectins and angiogenins, all of which have broad-spectrum or Gram-type

specific antibacterial activities (Ericksen *et al.*, 2005; Hornef *et al.*, 2004; Pellegrini *et al.*, 1992; Harwig *et al.*, 1995; Vaishnava *et al.*, 2011; Hooper *et al.*, 2003). Although Paneth cells first appear during prenatal gestation, their numbers and AMP secretion increase *post-partum* (Mallow *et al.*, 1996; Bry *et al.*, 1994). The expression of some AMPs is constitutive whereas others are dependent on colonization by the microbiota (Putsep *et al.*, 2000; Hooper *et al.*, 2003). This age-dependent augmentation of innate immunity in the neonatal intestine may be critical with respect to *E. coli* K1 infection. Experimental ablation of Paneth cells with the dye dithizone renders neonatal rats susceptible to intestinal overgrowth of the pathogen and dithizone-treated neonates have higher mortality rates than their untreated counterparts (Sherman et al, 2005).

The final major lineage of enteric epithelial cells is the goblet cell. These cells are present throughout the intestines but increase in number from the proximal duodenal compartment, where they comprise approximately 4% of the epithelium, to the distal colonic compartments, where they form around 16% of total epithelial cells (reviewed by van der Flier & Clevers, 2009). Goblet cells are secretory cells; they produce and maintain a fundamental component of the innate intestinal defensive mechanism: the intestinal mucus layer. The mucus layer has a well-established cytoprotective role in the gut and it has recently been demonstrated that, in addition to its capacity to inhibit bacterial adhesion to the epithelium, it forms a deep, stratified exclusion barrier which maintains the microbiota at a safe distance from the epithelial surface (Johansson *et al.*, 2008). The stratified layer is composed of gel-forming mucin proteins, such as Muc2, which are large linear glycoproteins polymerized in goblet cells by disulphide-bonded C-terminal dimerization and N-terminal trimerization, then secreted into the intestinal lumen where they form dimers with adjacent mucins through internal CysD domains (Ambort *et al.*, 2011; reviewed by Perez-Vilar & Hill, 1999). These interactions allow gel-forming mucins to produce the stratified exclusion barrier of the inner mucous layer. External to this layer and resting upon it is a much looser outer layer of mucus derived from the inner layer and colonized by the microbiota. The mucus layer is thinner in the small intestine and does not form an exclusion barrier, but serves as a repository for Paneth cell-derived AMPs (Vaishnava *et al.*, 2011; reviewed by Johansson & Hansson, 2011). Goblet cells also secrete trefoil factor peptides (Podolsky *et al.*, 1993); these are small proteins that bind to mucins and alter their viscoelastic properties. They appear to stabilize and maintain the function of the mucus exclusion barrier (Thim *et al.*, 2002,

Kindon *et al.*, 1995). Goblet cells and their secreted mucins appear in the intestine at an early stage of prenatal gestation, are not fully developed at birth and continue to proliferate postnatally (Chambers *et al.*, 1994; Fanca-Berthon *et al.*, 2009). The ontogeny of the colonic mucin exclusion barrier has not been investigated so it is not known when the barrier is formed. Interestingly, the secretion of the trefoil factor Tff3 occurs late in gestation and increases postnatally (Lin *et al.*, 1999, Mashimo *et al.*, 1995), indicating that the mucin-barrier function of the neonate may not be fully developed at birth, so it may influence susceptibility to pathogens such as *E. coli* K1.

The intestine contains a large amount of foreign antigenic material which, if allowed to come into contact with extra-intestinal tissues, would trigger a strong an immediate inflammatory response mediated by the systemic leukocyte populations. The adult intestine is the largest reservoir of macrophages in the mammalian body (Lee *et al.*, 1985) but displays a dulled pro-inflammatory cytokine production upon antigenic stimulation. This is due in part to the lack of expression of innate response receptors such as CD14 by intestinal macrophages, even though these macrophages maintain their capacity to phagocytose and eliminate invading bacteria (Smythies *et al.*, 2005). This inflammatory anergy has evolved to enable intestinal tissues to tolerate the antigenic load whilst undertaking vital absorptive functions in the absence of deleterious inflammatory reactions. Critically, this tolerance does not develop until the perinatal period (Maheshwari *et al.*, 2011) and has been shown to be developmentally regulated by exposure to foreign antigens such as LPS immediately *post-partum* (Lotz *et al.*, 2006). The macrophages and intestinal epithelial cells of very young and preterm neonates do not possess the non-inflammatory phenotype of older neonates. This may play a role in the development of NEC (Maheshwari *et al.*, 2011) and indicates that the immature bowel is susceptible to inflammatory damage, a factor which may be relevant to the intestinal translocation of *E. coli* K1 and other neonatal bacterial pathogens.

1.5 Aims & Objectives

The endogenous host tissues and the exogenous microbiota are in a state of developmental flux in the neonatal intestine and are likely to play a role in the determination of susceptibility of the neonate to at least some infections, in all likelihood including *E. coli* K1. Colonization of the intestine by *E. coli* K1 is age-independent. However, translocation across the GI epithelium and subsequent development of systemic disease is an age-dependent process. Insights into the influences of the developing microbiota and host tissues influences on the capacity of *E. coli* K1 to cause systemic disease in the neonate will shed light on the pathogenic mechanisms which drive the development of neonatal sepsis and meningitis. Moreover, a deeper understanding of these mechanisms may provide a rationale for the development of prophylactic strategies to control these often devastating infections and further the global campaign to reduce infant mortality in the 21st Century.

The primary aim of this project is to determine the influence of the developing intestinal microbiota and maturing intestinal tissues on the capacity of *E. coli* K1 to translocate from the neonatal intestine into the systemic circulation using a neonatal rat model of infection. Such work will contribute to the understanding of *E. coli* K1 pathogenesis, provide insights into the processes that drive the progression of the infection and may facilitate prophylactic interventions through abrogation of the capacity of the pathogen to egress from the GI lumen.

The initial objectives will be to develop the animal model and the methods required to fulfil to specific aims of the project. These include defining the age-dependency of *E. coli* K1 infection in the neonatal rat, thereby delineating susceptible and refractive neonatal populations for later analysis. The potential of the natural maternal-neonatal route to establish infection will be investigated, as will the design and optimization of an assay for the quantification of *E. coli* K1 in the intestinal microbiota.

The second objective will examine, using quantitative and qualitative analytical methods, the intestinal microbiota of neonates that are innately susceptible or refractive to systemic *E. coli* K1 infection. The dynamics of *E. coli* K1 intestinal colonization will be investigated in susceptible and resistant neonates. Any protective effect of the

microbiota will be determined by antimicrobial suppression of the natural microbiota of refractive neonates and by assessment of the impact of suppression on susceptibility to *E. coli* K1.

The final objective will examine the role of host intestinal tissues in the determination of susceptibility to *E. coli* K1 infection. Host tissue responses to *E. coli* K1 colonization will be determined at the transcriptomic level and the responses of susceptible and refractive neonatal tissues compared. Differentially expressed host factors of interest will be examined in greater depth, in terms of normal developmental expression and differential expression in response to *E. coli* K1 colonization. If appropriate and feasible, the mechanistic basis of differential expression will be explored.

CHAPTER 2

MODEL & METHOD DEVELOPMENT

2.1 Introduction

The use of animal models of infection remains a key element for the investigation of microbial pathogenesis and the development of new agents and modalities for the prophylaxis and treatment of infectious disease. In many cases, advances in *in vitro* technologies such as developments in organ culture, have not obviated the need for modelling infections in suitable animal hosts. Although the ability to grow different types of cell and even whole tissues in the laboratory environment has proven to be extremely useful in the study of host-pathogen interactions, these *in vitro* models can provide only preliminary evidence of the mechanics of *in vivo* interactions and hypotheses based on *in vitro* data must be validated *in vivo*. The rationale for this is clear; the different cell types and tissues of the multicellular organism are never found in isolation *in vivo* and are subject to modulation by endocrine, paracrine and nervous signalling which can have profound effects on the phenotype of a specific tissue, or of individual cells within that tissue. Furthermore, many systems, such as the digestive system and the GALT, are intrinsically interwoven with the lymphatic system. Thus, host-pathogen interactions are a complex interplay of factors, with specific interactions between the pathogen and host tissues that trigger systemic responses that cannot currently be thoroughly replicated *in vitro*.

Animal models for the study *E. coli* K1 infection generally employ rodent species, most frequently the laboratory rat *Rattus norvegicus* (Glode *et al.*, 1977; Bortolussi *et al.*, 1978; Kim *et al.*, 1992; Sukumaran *et al.*, 2003; Zelmer *et al.*, 2008) and the laboratory mouse *Mus musculus* (Pluschke & Pelkonen, 1988; Mittal *et al.*, 2010; Mittal & Prasadarao, 2011). Both species have a proven track record in replicating many features of infections in humans. Murine models present the investigator with a significant advantage that derives from the extensive genetic database that has been accumulated, together with the huge range of gene knockout (KO) strains that are available. However, the small size of the neonatal mouse sometimes presents a challenge; modelling *E. coli* K1 systemic infection may involve administration by the oral route and this can be problematical in this species. Conversely, although the rat lacks the powerful genetic capacity of the mouse, the relative size of the neonate allows for easier infection via the 'natural' oral route. There are also some differences in the innate immunity, specifically the expression of

defensins of both species compared to humans which may impact on their suitability as models for *E. coli* K1 infection. Firstly, the α-defensin repertoire of mice is much larger (20) than the rat (13) or human (9) and rat defensins are in the main more closely related to humans that the murine equivalents (Patil *et al.*, 2004). Secondly, murine neutrophils do not express defensin peptides, whereas both rat and human neutrophils do (Eisenhauer *et al.*, 1992), which may be of importance considering the likely role of these cells in *E. coli* K1 pathogenesis (Mittal & Prasadarao, 2011). These differences indicate that the rat is almost certianly more suitable in terms of modelling human infections than the mouse.

The anatomical configuration of the GI tract of humans and rats is similar but there are some key structural differences between this organ in these two species (reviewed by Kararli, 1995). The caecum of the rat is enlarged in comparison to that of humans and rats lack a gallbladder. The rat secretes bile salts directly into the small intestine from the liver *via* the hepatic bile duct but in most mammals, including humans, bile salts are concentrated in the gallbladder prior to secretion. However, key elements of the biochemistry of the GI tract, such as pH and bile salt concentration and composition, are comparable. Diseases of the GI tract that afflict humans and are considered to possess a major microbial aetiological component, such as NEC, can be reproduced experimentally in the rat and the symptoms displayed often closely mirror those of the human condition (Caplan *et al.*, 2001), Rotavirus (Ciarlet *et al.*, 2002) and *Salmonella* infections (Naughton *et al.*, 1996) provide good examples. GI tract colonization of susceptible neonatal rats after oral administration of *E. coli* K1 produces an infection which closely mimics the course of human sepsis and meningitis; the bacteria disseminate into the blood compartment, gain access to systemic tissues and can induce lethal inflammatory responses in the CNS (Glode *et al.*, 1977; Zelmer *et al.*, 2008; 2010). This evidence strong suggests that the neonatal rat is an appropriate model animal for the study of *E. coli* K1 age dependent pathogenesis.

The majority of laboratory rats currently in use are derived from the outbred Wistar strain developed for use as a general model organism in the early 20th century. These animals were selectively bred to maintain traits useful to researchers such as docility and the ability to thrive in a laboratory environment. Many strains have been derived from the Wistar rat for use in different research areas. Examples include Long Evans and Zucker rats (obesity), Sprague Dawley rats (general research, toxicology and

oncology) as well as Athymic Nude and Fischer 344 rats (immunology). Previous studies which have employed a rat model of *E. coli* K1 infection have used the general research Wistar and Sprague Dawley rat strains. In our laboratory the Wistar strain has previously been successfully used to study different aspects of *E. coli* K1 infection including experimental chemotherapy (Mushtaq *et al.*, 2004; 2005; Zelmer *et al.*, 2010) and pathogenesis (Zelmer *et al.*, 2008). This model was therefore employed in this investigation.

The epidemiology of *E. coli* K1 infection persuasively infers that acquisition of the pathogen by the neonate generally occurs by vertical transmission from the maternal intestinal and/or vaginal microbiota during the perinatal period, although secondary non-maternal acquisition from the environment does occur (Sarff *et al.*, 1975; Glode *et al.*, 1977). However, there have been few, if any, attempts to replicate vertical transmission in animals. Oral challenge models of infection employ bacterial innocula of 10^5-10^8 CFU *E. coli* K1 (Glode *et al.*, 1977; Mushtaq *et al.*, 2005; Zelmer *et al.*, 2008; 2010), probably a much larger than that encountered in the natural infection. Further, this mode of experimental infection does not take into account any phenotypic variation between laboratory-cultured bacteria and those of the maternal GI tract.

Quantification of bacteria in experimental infections presents a number of challenges, particularly in a heavily contaminated environment such the GI tract, which possesses a large resident bacterial population. Many intestinal bacteria are difficult to grow on laboratory media and traditional culture methods are limited in their capacity to discriminate between members of the microbiota and the bacterial innoculum. Selective media containing antibiotics or other inhibitors may be used to aid discrimination, as will the inclusion of a pH indicator that responds to specific bacterial metabolites. For differentiation of *E. coli* from other, related bacteria, MacConkey agar, containing inhibitory bile salts and toluene red as a pH indicator, is frequently employed for the detection of *E. coli* within complex bacterial populations. Further differentiation of *E. coli* K1 from other *E. coli* clones can be achieved using K1-specific lytic bacteriophage (Gross *et al.*, 1977; Cross *et al.*, 1984).

An *E. coli* K1-specific quantitative polymerase chain reaction (qPCR) would provide a viable alternative to culture and phage-typing methods. PCR is widely used as a molecular diagnostic tool for the detection of a wide range of pathogens (reviewed by

Malorny *et al.*, 2002); qPCR was developed to achieve real-time monitoring using DNA-binding fluorophores and permits quantification of the copy number of target DNA sequences by comparison of the PCR amplification curves from DNA sample amplification with those from amplification of known quantities of target DNA (for example Palmer *et al.*, 2007; Furet *et al.*, 2004; Gueimonde *et al.*, 2004). It may provide a valuable tool for assessing colonization of the GI tract by *E. coli* K1.

Here I describe the development of the neonatal rat model of systemic *E. coli* K1 infection and introduce analytical methods for the investigation of intestinal colonization by *E. coli* K1.

2.2 Materials & Methods

Unless otherwise indicated, media for bacterial cultivation were purchased from Oxoid Ltd, chemicals, reagents and enzymes were purchased from Sigma-Aldrich and oligonucleotides were synthesised by, and purchased from, Eurofins MWG Operon.

2.2.1 Bacteria: strains, growth conditions and stock maintenance

The bacterial strains used throughout this work are shown in Table 2.1. *E. coli* K1 strain A192PP is a derivative of strain A192 (also designated DSM 10719 in the Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ] collection), a neonatal septicaemia isolate from the Netherlands and described by Achtman *et al.* (1983). The virulence of A192 was enhanced by serial passage in the neonatal rat by

Strain	Description	O:K serotype	Source
A192PP	Enhanced virulence; septicaemia isolate	O18:K1	Mushtaq *et al.*, 2004
C14	UTI isolate	O?:K1	In-house collection
DSM 10723	Meningitis isolate	O18:K1	DSMZ
LP1674	UTI isolate	O7:K1	In-house collection
EV36	K-12/K1 hybrid	O?:K1	Vimr & Troy, 1985
LP1395	UTI isolate	O18:K?	In-house collection
DSM 10797	UTI isolate	O18:K5	DSMZ
DSM 10794	UTI isolate	O18:K5	DSMZ
CGSC 5073	K-12 strain	N/A	CGSC
Klspp10	*Klebsiella pneumoniae* isolate	N/A	In-house collection
Citro14	*Citrobacter freundii* isolate	N/A	In-house collection
Prmirab42	*Proteus mirabilis* isolate	N/A	In-house collection

Table 2.1: *Bacteria used in this study. Strain designations, descriptions and O-/K-antigenic serotypes are provided where applicable. Strains were obtained from an in-house collection at the UCL School of Pharmacy or purchased from either the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) or the Coli Genetic Stock Centre (CGSC). All strains are E. coli unless indicated.*

Mushtaq *et al.*, (2004); a single colony from the blood culture of an infected rat after second passage was designated A192PP. This strain efficiently colonizes the GI tract and causes systemic infections in 100% of susceptible neonatal rats. It was used in this study for all oral challenges with *E. coli* K1. Strain EV36 is an *E. coli* K-12 strain carrying the *E. coli* K1 RS1085 *kps* locus on an Hfr plasmid; this locus encodes the genes for K1 capsule biosynthesis (Vimr & Troy, 1985). Bacteria were grown in Mueller-Hinton (MH) broth in an orbital incubator (200 orbits/min) and on MH or MacConkey agar; cultures were incubated overnight (~24 h) at 37 °C. Optical density of liquid cultures was measured at a wavelength of 600 nm (OD_{600}) using a Lambda 25 spectrophotometer (Perkin-Elmer). Stocks of each strain were prepared by mixing aliquots of liquid cultures with sterile glycerol to a final glycerol concentration of 20% (v/v) and stored at -80 °C.

2.2.2 Animals

Pregnant, non-pregnant and lactating adult Wistar rats with neonatal pups were purchased from Harlan Olac UK. All adult rats were 9-11-week-old females and were housed in individual cages with associated neonates. Neonates were of mixed gender and either provided with their natural mothers or littered in-house, in the case of pregnant animals. Pregnant animals were supplied at 12-14 days gestation. All animals were kept in rooms at 19-21 °C with 45-55% humidity, 15-20 air changes/h, and a 12 h light/dark cycle. They were provided with a 5LF5 basic maintenance diet and water *ab libitum*. Adults were killed by CO_2 euthanasia and neonates by decapitation. All procedures conformed to National and European legislation and were approved by the institutional Ethics Committee and the UK Home Office.

2.2.3 Bacteriophage K1E propagation, purification and titration

E. coli K1-specific lytic bacteriophage K1E was isolated by Gross *et al.* (1977), has been further characterized by Tomlinson & Taylor (1985) and Leiman *et al.* (2007) and was provided by Tom Cheasty (Health Protection Agency, UK). Methods for bacteriophage propagation precipitation were based on those described by Tomlinson &

Taylor (1985). Five hundred mL cultures of A192PP were grown to OD_{600} 0.8, K1E bacteriophage added at a multiplicity of infection (MOI) of 0.25 and the mixture incubated for a further 30 min at 37 °C. Cultures were cooled to room temperature, DNase I and RNase added to 1 µg/mL and left to stand for 30 min. NaCl was added to give a final concentration of 1 M and the culture placed on ice for 1 h. PEG 8000 was added to give a final concentration in solution of 10% (w/v). Cultures were maintained in ice water for 1 h. Precipitated phage particles were recovered by centrifugation at 11000 x *g* for 10 min and the pellet suspended in SM buffer (100 mM NaCl, 8 mM $MgSO_4 \bullet 7H_2O$, 50 mM pH 7.5 Tris-HCl). PEG and cellular debris were extracted by addition of an equal volume of chloroform followed by gentle mixing and centrifugation at 3000 x *g* for 15 min to recover the aqueous phase containing phage. The phage suspension was filtered (0.22 µm MILLEX GP filter; Millipore) and the filtrate stored at 4 °C. Phage was titrated using ten-fold serial dilutions of the filtrate and each diluted suspension incubated for 5 min with mid-exponential-phase (OD_{600} 0.5) A192PP in 3 mL of molten overlay agar (0.5% [w/v] Bacteriological agar in MH broth). Overlay agar was spread onto MH agar base and incubated overnight at 37 °C. Plaques were enumerated and expressed as plaque forming units per mL (PFU/mL).

2.2.4 Oral inoculation of neonates and adults

The inoculation of neonatal rats by the oral route was based on a method developed by Glode *et al.* (1977) and refined by Pluschke *et al.* (1983). *E. coli* strains were grown in liquid culture to mid-exponential-phase (OD_{600} 0.5; 0.5 x 10^9 CFU/mL); 2-9 day old (P2-P9) neonatal rats were removed from their cages and fed 20 µL of a bacterial suspension (10^7 CFU) using a micropipette fitted with sterile tips pre-warmed to 37 °C. Controls received sterile MH broth. Animals were returned as soon as possible to their cages. Pregnant and non-pregnant adult rats were given larger amounts of mid-exponential-phase bacteria (10^8-10^{10} CFU) by gastric lavage.

2.2.5 Processing of tissue & stool samples

Neonatal tissue and adult stool samples were collected for downstream analysis. For collection of adult stool samples, rats were removed from their cages and placed in a clean cage without bedding. Fresh stools were obtained immediately post-defaecation and placed in pre-weighed collection tubes containing 4 mL ice-cold phosphate buffered saline (PBS) and kept on ice. Neonatal whole intestinal (duodenum-rectum) tissues were collected by dissection of animals immediately *post-mortem*. Tissue removal was performed under sterile conditions in a class II biological safety cabinet (C2BSC) with sterile instruments. These were soaked in 70% ethanol and washed with sterile PBS between samples. Tissues for bacterial enumeration and DNA extraction were placed in pre-weighed collection tubes containing 2 mL ice-cold PBS and kept on ice. After collection, all samples were immediately weighed and homogenized on ice using an Ultra-Turrax T-10 homogenizer (IKA-Werke). The homogenizer blade was washed before homogenization of each sample with 70% ethanol followed by three washes in sterile PBS. Tissue and stool homogenates were examined immediately or stored at -80 °C.

2.2.6 Detection of *E. coli* K1 colonization and bacteraemia

Inoculated neonatal rats were examined for intestinal colonization by *E. coli* K1 and the presence of bacteria in blood. Intestinal colonization was determined after peri-anal swabbing. Sterile swabs were moistened in sterile PBS and used to swab the neonatal peri-anal area; swab tips were placed in Eppendorf tubes containing 300 µL sterile PBS, the tube contents mixed by vortex for 30 s and 100 µL spread-plated onto MacConkey agar and incubated overnight at 37 °C. Blood (10 µL) was taken from the foot pad with a 26G BD microlance and blood mixed with 90 µL PBS containing heparin at a concentration of 2 units/mL. The mixture was plated onto MacConkey agar; after overnight incubation at 37 °C, plates were examined for coliform lac^+ (pink) colonies. Pink colonies were examined for susceptibility to *E. coli* K1-specific phage. Colonies were picked with a sterile loop, placed in 200 µL sterile PBS, mixed and streaked onto MH agar plates. Streaks were left to dry and 10 µL of 10^9 PFU/mL K1E phage suspension dropped onto the mid-point of each streak; plates were incubated

overnight at 37 °C. After incubation, bacterial streaks were examined for phage lysis to determine the presence or absence of K1 capsule (Figure 2.1). Phage-sensitive ($K1^+$) bacteria were assumed to be *E. coli* K1.

2.2.7 *E. coli* K1 quantification

E. coli K1 from neonatal tissue and adult stool samples were quantified in similar fashion to the method to that described in 2.2.6 (Figure 2.2). Serial tenfold dilutions (100 µL) of tissue and stool homogenates were spread-plated on MacConkey agar and incubated overnight at 37 °C. Coliform colonies were sub-cultured on MH agar and their sensitivity to K1E phage determined. After enumeration, the data was normalized to sample mass to give CFU/g tissue or stool.

2.2.8 DNA extraction

Bacterial DNA was extracted for non-quantitative PCR and for use as genomic standards in qPCR. Strains were streaked onto MH agar from glycerol stocks and incubated overnight at 37 °C. Isolated colonies were used to inoculate 10 mL MH broth and grown to OD_{600} 0.5 and bacteria recovered by centrifugation at 5000 x *g* for 10 min. A192PP provided qPCR genomic standards: serial dilutions were plated in triplicate onto MH agar, incubated overnight at 37 °C and enumerated by colony plate count. Total DNA was extracted from bacterial pellets using QIAamp DNA Mini kits (Qiagen) according to the manufacturer's instructions. The composition of extraction buffers is proprietary information unless otherwise stated. Pellets were suspended in 180 µL of lysis buffer ATL supplemented with 20 µL proteinase K (20 mg/mL) and incubated at 56 °C for 1 h. RNA was selectively degraded by addition of 4 µL RNase A (100 mg/mL) and incubated at room temperature for 2 min; 200 µL lysis buffer AL was added to the DNA extraction mixture, mixed by vortex for 15 s and incubated at 70 °C for 10 min. Ethanol (100% [v/v]; 200 µL) was added, the tube contents mixed by vortex for 15 s and applied to a QIAamp Mini spin-column. The column was centrifuged at 6000 x *g* for 1 min. The filtrate was discarded and the column washed with 500 µL each of the wash buffers AW1 and AW2. Elution buffer AE (10 mM pH 9 Tris-HCl, 0.5 mM

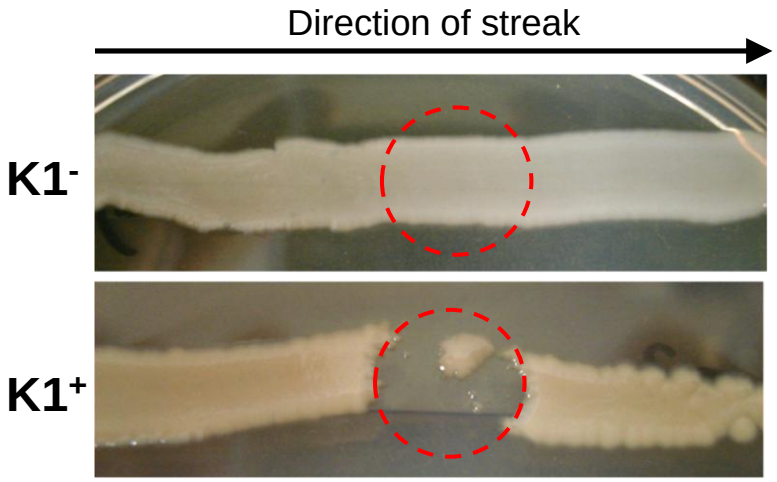


Figure 2.1: *Identification of K1 capsule by K1E bacteriophage-mediated lysis ($K1^+$) of coliform bacteria. Red circles indicate area covered by phage droplet.*

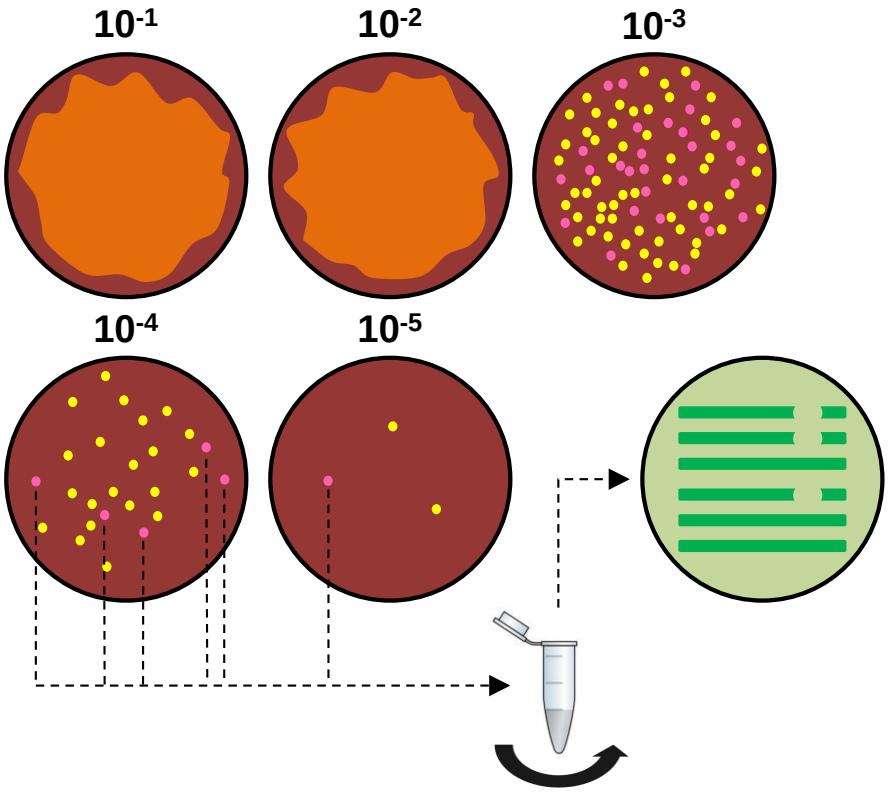


Figure 2.2: *E. coli K1 quantification by culture and phage-typing. Tenfold dilutions, MacConkey agar (red), Mueller-Hinton agar (light green), confluent bacterial growth (orange), lac^- colonies (yellow), lac^+ colonies (pink) and sub-cultured bacterial streaks (green) are illustrated.*

EDTA; 200 µL) was applied to the column and the DNA eluted by centrifugation at 6000 x *g* for 1 min. This procedure was repeated using a further 200 µL of buffer AE to ensure full recovery of the DNA. The concentration and purity of DNA were determined with a NanoDrop spectrophotometer (Thermo Scientific). The genome copy number (gDNA/µL) was calculated using plate counts. DNA samples were stored at -20 °C.

2.2.9 DNA extraction of GI tissues and stool samples

DNA was extracted from neonatal GI tract tissues, with contents, and adult stool samples for downstream PCR. Tissue and stool extractions were undertaken using QIAamp DNA Stool Mini kits (Qiagen) according to the manufacturer's instructions. The composition of buffers or tablets was proprietary information unless otherwise indicated. Initially, 200 µL of tissue or stool homogenate was mixed with 1.4 mL of lysis buffer ASL, mixed by vortex and incubated at 95 °C for 5 min. Extraction mixtures were again mixed by vortex for 15 s and centrifuged at 20000 x *g* for 1 min to pellet tissue debris or stool particles. An InhibitEx tablet was then added to 1.2 mL of the recovered supernatant and mixed by vortex until the tablet was completely suspended. The tablet was pelleted by centrifugation at 20000 x *g* for 3 min. 200 µL of the supernatant was collected and again centrifuged to remove any further suspended tablet material. Proteinase K (20 mg/mL; 15 µL) was added to the 200 µL extraction mixture followed by 200 µL of the lysis buffer AL; after mixing by vortex for 15 s, the mixture was incubated at 70 °C for 10 min. Ethanol (100% [v/v]; 200 µL) was added to the lysate and the mixture applied to a QIAamp spin-column. The columns were centrifuged at 20000 x *g* for 1 min. The filtrate was discarded, the columns washed sequentially with 500 µL of wash buffers AW1 and AW2 and dried by centrifugation at 20000 x *g* for 1 min. Elution buffer AE (10 mM pH 9 Tris-HCl, 0.5 mM EDTA; 200 µL) was applied to the column and DNA eluted at room temperature as described above. The concentration and purity of DNA were determined with a NanoDrop spectrophotometer (Thermo Scientific).

2.2.10 *neuS* PCR and amplicon agarose gel electrophoresis

The *E. coli* K1-specific gene *neuS* encodes poly-α-2, 8 sialosyl sialyltransferase and was selected the development of a PCR assay for the identification and quantification of E. coli K1 in mixed bacterial populations. The oligonucleotide primer pair NeuSF3 (5'-CCAA AGAAGATGATGTTAATCCAATTAAG-3') and NeuSR3 (5'-ATCATCAACCAGAATAGATAATGTTATCC-3') was designed to amplify a 332 bp fragment within the *neuS* gene. The primer pair was designed using v.9 Clone Manager Suite software (Scientific & Educational Software) utilizing the *neuS* gene sequence from the O7:K1 serotype NMEC strain CE10 complete genome sequence (NCBI accession number: NC_017646) as a design template. Primer specificity for *E. coli* K1 strains was examined using Primer-BLAST (www.ncbi.nlm.nih.gov /tools/primer-blast) with primer pair specificity checking parameters set to all deposited bacterial and *Rattus norvegicus* sequences in all DNA sequence repository databases. PCR reactions (50 µL) were prepared by mixing 25 µL of GoTaq Green Master Mix (Promega) with 10 µL nuclease-free water, 5 µL each of 2.5 µM NeuSF3 and NeuSR3 primers (final primer concentration 625 nM) and 5 µL of 40 ng/µL bacterial DNA (200ng in total). PCR reactions were performed in a Techne Thermocycler (Bibby Scientific). The thermocycling program comprised an initial DNA denaturation step of 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 61 °C for 30 s and amplicon extension at 72 °C for 30 s and a final extension cycle at 72 °C for 5 min. Amplified DNA was resolved by loading 10 µL of PCR reaction mixture onto a 1% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide and electrophoresis at 80 V in Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate, 1mM EDTA, pH8) for 30 min or until the dye front reached the end of the gel. Ethidium bromide-intercalated DNA within the gel was visualized by scanning with a Molecular Imager FX system (Bio-Rad) set to detect UV fluorescence.

2.2.11 Amplicon *cleanup* and DNA sequencing

PCR products amplified with GoTaq Green Master Mix were cleaned using Wizard SV Gel and PCR *Cleanup* kit (Promega) according to the manufacturer's instructions prior to DNA sequencing. PCR reaction mixture (20 µL) was mixed with

20 µL of Membrane Binding Solution (4.5 M guanidine isothiocyante, 0.5 M potassium acetate, pH 5.0). The mixture was then transferred to a *cleanup* kit spin column and incubated at room temperature for 1 min to allow binding to the membrane. The column was centrifuged at 16000 x *g* for 1 min and the filtrate discarded. The membrane was washed with 700 µL followed by 500 µL of Wash Solution (10 mM potassium acetate pH 5.0, 80% ethanol, 16.7 µM EDTA, pH 8.0), with centrifugation at 16000 x *g* for 1 min after each wash. The wash filtrate was discarded and the membrane dried by centrifugation of the column at 16000 x *g* for 5 min. Nuclease-free water (50 µL) was applied to the column membrane and DNA eluted by incubation at room temperature for 1 min followed by centrifugation at 16000 x *g* for 1 min. Cleaned DNA was assayed for concentration and purity using a NanoDrop spectrophotometer (Thermo Scientific) and stored at -20 °C. DNA for sequencing was prepared in Eppendorf tubes in 15 µL volume containing 5 ng/µL cleaned DNA and the primer NeuSF3 at a final concentration of 15 pM. DNA sequencing was undertaken by Eurofins MWG Operon; sequences were aligned with the *neuS* sequence using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2) to verify sequence identity.

2.2.12 *E. coli* K1 quantification by *neuS* qPCR

A qPCR assay of the *neuS* gene was used to quantify *E. coli* K1 in tissue or stool samples by DNA analysis. Genomic standard DNA was prepared by 10-fold serial dilution of A192PP DNA using previously calculated gDNA/µL values (see section 2.2.8). Standards used in qPCR typically ranged from 2-2000 gDNA/µL. qPCR reactions (15 µL) were established by combining, in order and on ice, 2.7 µL of nuclease-free water, 10 µL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies), 1 µL each of 12.5 µM NeuSF3 and NeuSR3 primers (see section 2.2.10; final concentration per primer of 625 nM) and 0.3 µL of 600 nM ROX reference dye (final concentration 30 nM). qPCR reactions were prepared in light-protected tubes and in large batches, depending on the number of reactions required for each experiment and to ensure reaction mixture homogeneity. Preparation of qPCR reactions was also carried out in a C2BSC to reduce the risk of DNA contamination. Batch-made qPCR reaction mixtures were divided into individual 15 µL reactions by pipetting into 96-well PCR plates. 5 µL of genomic standard DNA, tissue or stool

sample DNA extracts, or nuclease free water (acting as a no-template control) were added to each qPCR reaction mixture to a final volume of 20 µL. Wells were sealed with optically clear strip caps. qPCR reactions were carried out using an Mx3000P system and v.2 software (Stratagene) set to detect SYBR1 and ROX fluorescence. The thermal cycling programme comprised an initial DNA denaturation step at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 20 s and anneal/extend at 61 °C for 20 s. Fluorescence was measured at the anneal/extend step of each amplification cycle and amplification curves recorded. DNA melt-curves to determine the number of DNA products produced during amplification were constructed by cooling reaction mixtures to 55 °C and incrementally increasing to 95 °C over 30 min; fluorescence was measured at 20 s intervals. SYBR1 fluorescence was normalized to ROX fluorescence to enable the software to generate a cycle-threshold (Ct) of SYBR1 fluorescence utilizing adaptive baseline and amplification-based threshold algorithm enhancements. Ct values of genomic standard DNA amplifications were used to generate standard curves for analysis of test DNA samples, enabling determination of sample concentration (gDNA/µL) and calculation of *E. coli* K1 CFU/g sample. Standard gDNA extracted from 3 separate A192PP cultures was used for each qPCR reaction plate and standards, samples and control reactions were duplicated on each plate. All assays were duplicated on a separate occasion and data for each sample averaged across the 4 replicate values.

2.3 Results

2.3.1 Characterization of the neonatal rat model of *E. coli* K1 infection

2.3.1.1 Age-dependency

The impact of age on susceptibility to lethal infection of was examined by feeding *E. coli* A192PP cultures to neonates 2-9 days *post-partum* (P2-P9). Sterile MH broth was used as a control. Experimental and control groups comprised two litters, each of twelve neonates, for each age-group. Survival of colonized neonates was monitored for two weeks after inoculation but no deaths were recorded after 7 days had elapsed; survival over this period was recorded and used to construct Kaplan-Meier survival curves (Figure 2.3). Animals manifesting symptoms of systemic disease, such as lack of responsiveness and pallor, were immediately culled and blood and brain tissue obtained in order to determine the presence of *E. coli* K1. The data demonstrates a strong correlation between the age of inoculation and survival in response to oral inoculation with A192PP. Analysis of the survival curves produced in this experiment indicated the presence of three distinct groups. P2-3 neonates were extremely susceptible to A192PP and had very low survival rates. P4-6 neonates were moderately susceptible to A192PP and had intermediate survival rates. P8-9 neonates were mostly refractive to A192PP and had high survival rates. No mortality was observed in any of the control litters and *E. coli* K1 was found in blood and brain samples from culled sick animals (n=7) using culture and K1E phage-typing methods, strongly indicating that A192PP was the aetiological agent of mortality.

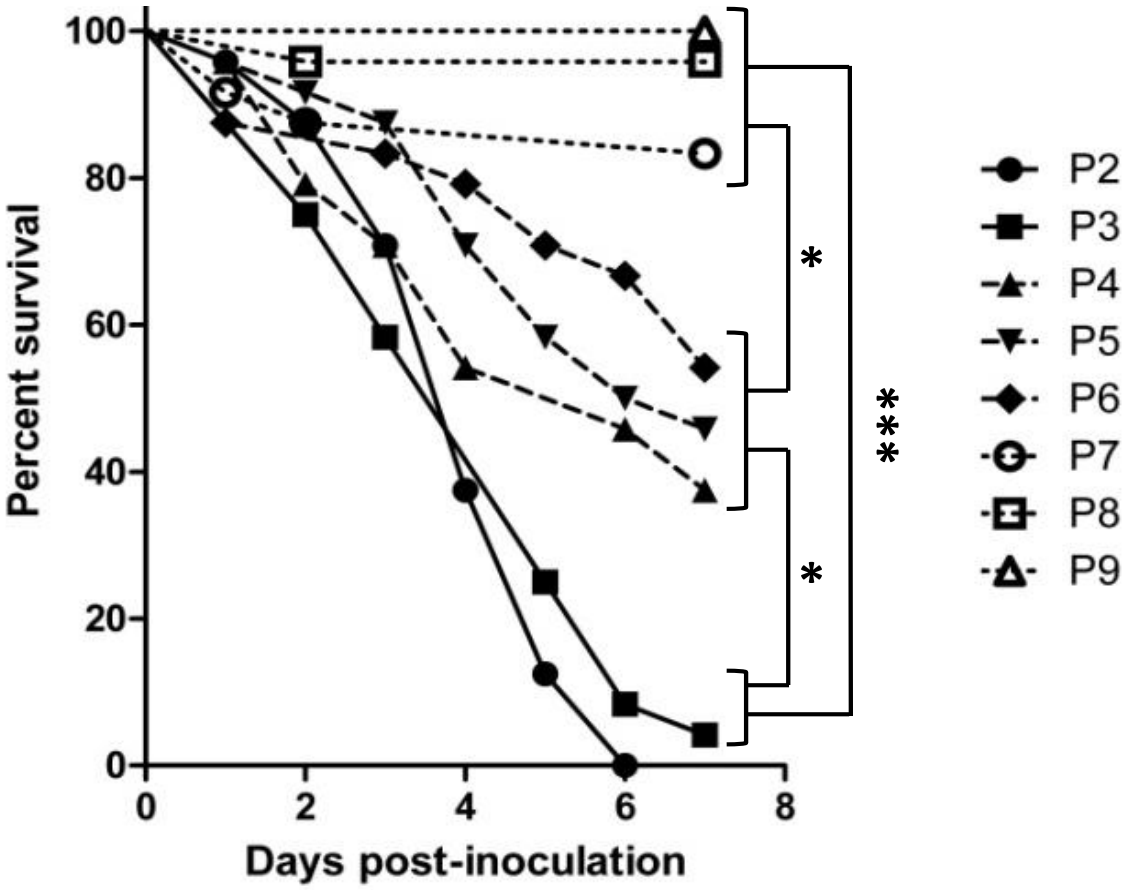


Figure 2.3: *Age-dependent survival of neonatal rats in response to oral inoculation with E. coli K1. P2-P9 (n=24 per group) neonates were orally inoculated with 10^7 CFU of strain A192PP and survival monitored for seven days. Significant differences in survival as determined by Logrank test are indicated (* p<0.05, ** p<0.01, *** p<0.001).*

2.3.1.2 Relationship between colonization, bacteraemia and mortality

The susceptibility of P2, P5 and P9 neonatal rats to *E. coli* K1 was examined together with an assessment of intestinal colonization and translocation into the blood compartment. Litters of 12 neonates for each age group were inoculated with mid-exponential-phase liquid cultures of A192PP and monitored for survival over seven days. Each neonate was assessed daily during this period for intestinal colonization and bacteraemia by selective culture and K1E phage typing to determine the presence of *E. coli* K1. Deaths, colonization and bacteraemia were recorded for each age group, with

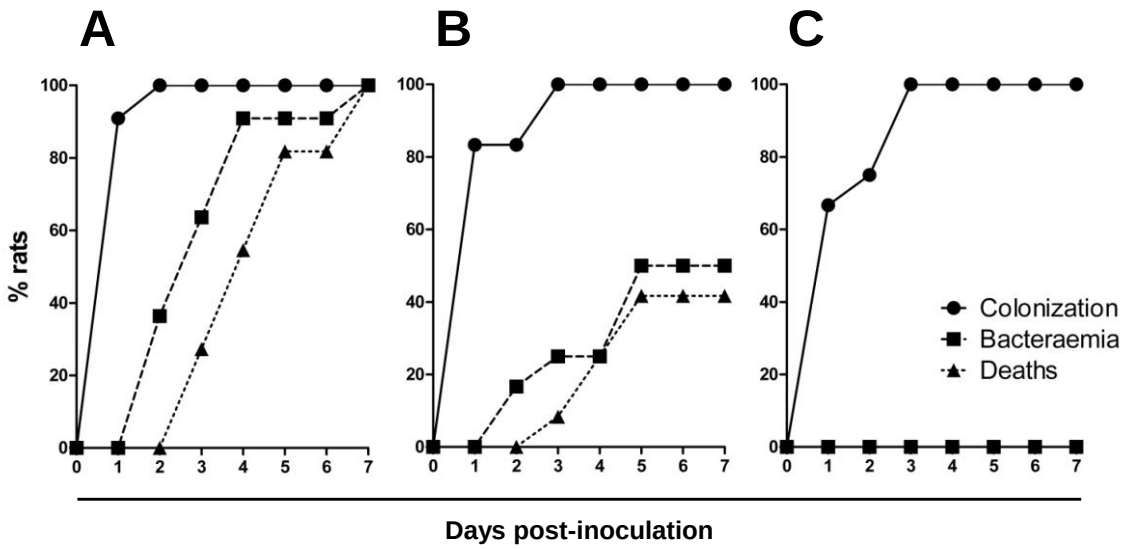


Figure 2.4: *Colonization, bacteraemia and deaths in neonatal rats orally inoculated with E. coli A192PP at P2 (A), P5 (B) and P9 (C). Litters of twelve neonatal rats were inoculated with 10^7 CFU and colonization and bacteraemia detected by culture and K1E phage-typing of peri-anal swabs and blood samples. Data are from two or more experiments.*

dead animals scored as colonized and bacteraemic (Figure 2.4). *E.coli* A192PP colonized neonates inoculated at P2, P5 and P9, although there was a small but noticeable lag in *E. coli* K1 detection in P9 and, to a lesser extent, P5-inoculated neonates. Mortality rates of the three age groups were comparable to those in Figure 2.3 and there was a strong correlation between bacteraemia and death, with a 94.4% incidence of mortality in animals with *E. coli* K1 bacteraemia. Although all rats inoculated at P9 were colonized 72 h after inoculation, no bacteraemia was detected in any of these animals. The lack of detectable bacteraemia in the refractive P9 neonatal group and the strong correlation between bacteraemia and mortality in the more susceptible P2 and P5 cohorts is a strong indication that the capacity of *E. coli* K1 to translocate from the intestines is the determining factor of the age-dependency of systemic infection.

2.3.1.3 Onset of systemic infection

The onset of systemic *E. coli* K1 infection in susceptible neonates was investigated using phage K1E to selectively reduce the *E. coli* K1 intestinal population at various time-points after inoculation with *E. coli* K1 (Figure 2.5). Litters of twelve P2 neonates were inoculated with 10^7 CFU of strain A192PP and intestinal colonization, bacteraemia and deaths recorded; animals which died were scored as colonized and bacteraemic. Neonates with each litter were orally inoculated with 10^9 PFU phage K1E at P3, P4 and P5, that is one, two, and three days after A192PP inoculation. Inoculation with K1E at all time points resulted in a significant decrease in the proportion of rats from which *E. coli* K1 intestinal colonization could be detected by peri-anal swabbing (Figure 2.5 A-C). The development of bacteraemia and subsequent mortality was significantly reduced in experimental groups which received the phage inoculum at P3 and P4 (Figure 2.5 A/B). However, neither were reduced in neonates that received the K1E inoculation at P5 (Figure 2.5 C) and this group suffered comparable rates of bacteraemia and mortality to SM buffer-inoculated controls (Figure 2.5 D). Although a small degree of mortality was observed in neonates inoculated at P4, this was attributable to neonates which were bacteraemic prior to K1E inoculation. The inability of phage to prevent mortality in colonized neonates inoculated with K1E at P5 strongly suggests that the onset of bacteraemia occurred prior to this time-point. As the proportion of bacteraemic rats in this group continued to rise after K1E administration, it is very likely that these animals were already bacteraemic but bacterial numbers in the blood were below the detection threshold.

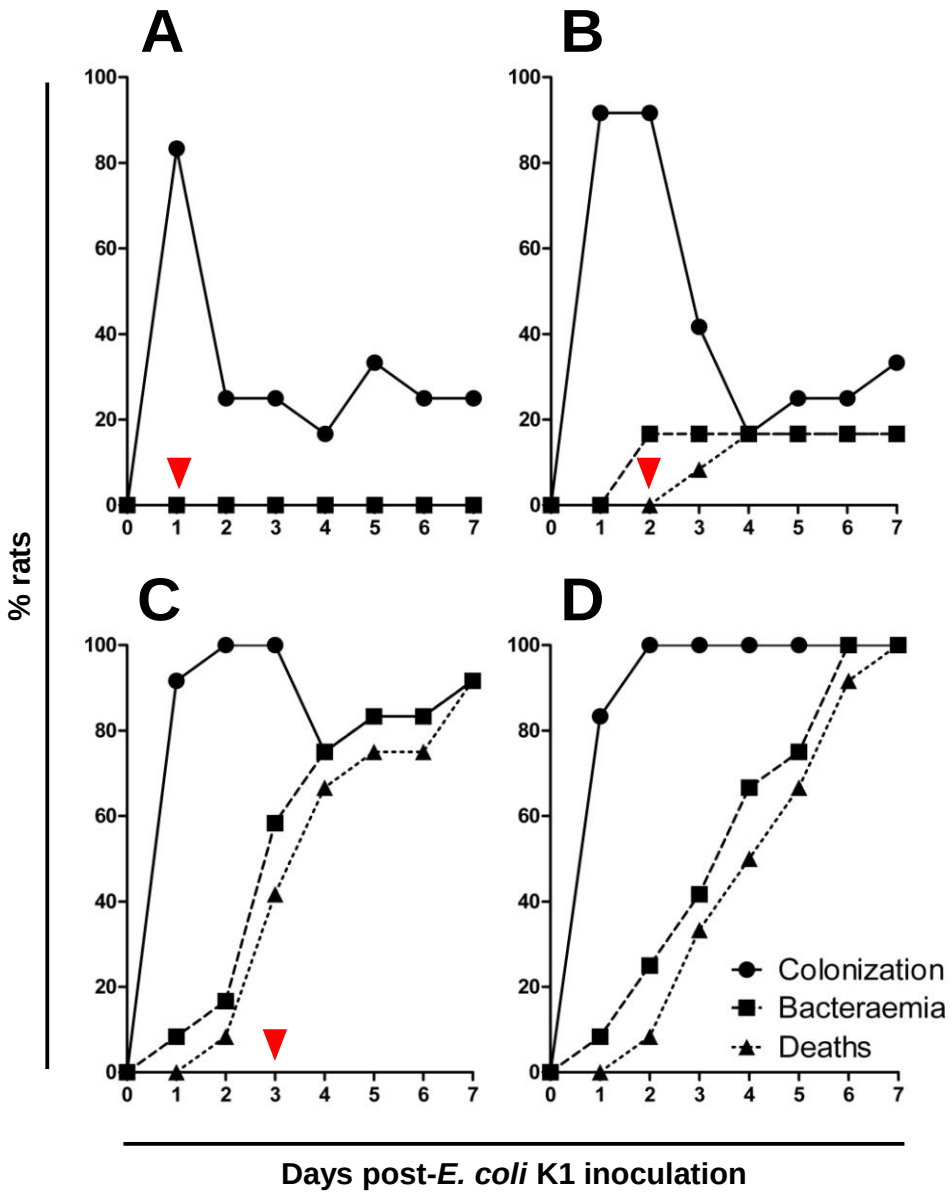


Figure 2.5: *Colonization, bacteraemia and deaths in P2 neonates colonized by E. coli K1 and inoculated with phage K1E. Neonates were fed 10^9 PFU of K1E (▼) at P3 (A), P4 (B) and P5 (C) or were treated with sterile SM buffer at P3 (D). Data are from two or more experiments.*

2.3.2 The maternal-neonatal route of infection

The vertical transmission of the *E. coli* A192PP from the pregnant mother to the neonate during the peri-natal period was investigated to determine the viability of this route of infection for further studies.

2.3.2.1 Colonization of adults rats with *E. coli* K1

Induction of stable colonization of the adult rat intestinal tract with *E. coli* A192PP was investigated by gastric lavage of non-pregnant adult rats. Rats were inoculated with 10^8, 10^9 or 10^{10} CFU or with sterile MH broth as a control.

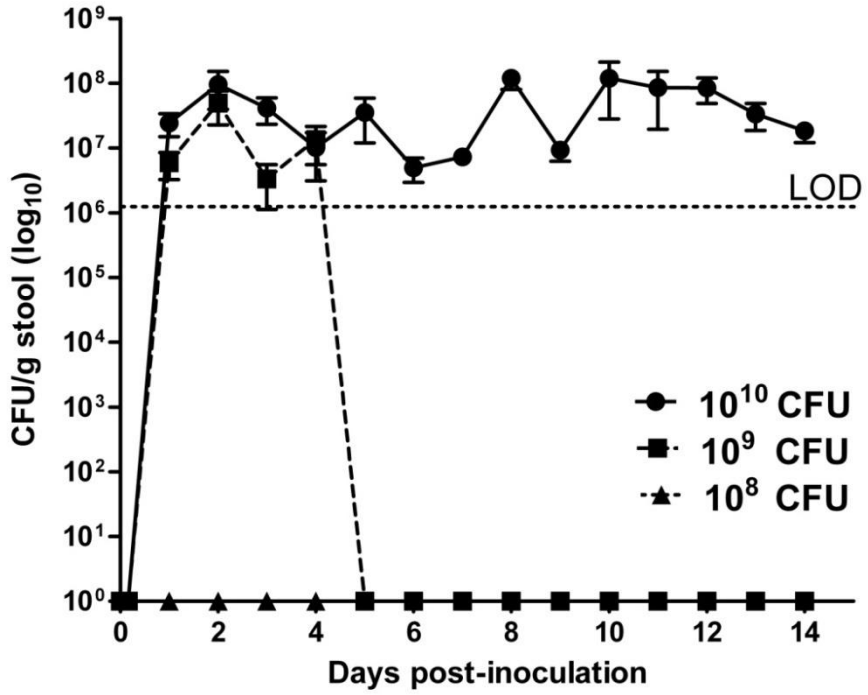


Figure 2.6: *Intestinal colonization of non-pregnant adult rats by E. coli A192PP. Rats were inoculated with 10^8, 10^9 or 10^{10} CFU by gastric lavage. Error bars represent the SEM of CFU/g quantified from n=3 rats. The limit of detection (LOD) is indicated.*

Each test group comprised three adults; two rats were employed for each control group. Two stool samples were collected from each animal over a fourteen day period after colonization and the presence of *E. coli* K1 determined (Figure 2.6). An inoculum of 10^{10} CFU A192PP was required to induce stable and prolonged *E. coli* K1 colonization of the adult intestine. Inoculation with 10^{9} CFU produced a transient colonization with *E. coli* K1 no longer detectable after five days. No *E. coli* K1 could be detected in the stool of animals inoculated with 10^{8} CFU or in control animals. The mean mass of stool samples (n=330) was 0.32 g and a sample dilution factor of 10^{-4} was required to culture individual *lac*$^{+}$ colonies for phage typing. The limit of detection (LOD) based on selective culture and phage typing was determined to be approximately 1.24×10^{6} CFU/g of stool.

2.3.2.2 Colonization of pregnant rats with *E. coli* K1

The method of induction of stable colonization in non-pregnant adult rats was applied to pregnant adults in order to investigate the feasibility of establishing neonatal colonization through maternal-neonatal vertical transmission. Four pregnant rats were inoculated with 10^{10} CFU A192PP and two with sterile MH broth as a control. Stools were collected and processed to determine *E. coli* K1 CFU/g of stool as described in the previous section and the survival of live offspring monitored *post-partum* (Figure 2.7). Pregnant rats were inoculated at thirteen days of gestation (E13) and the extent of *E. coli* K1 colonization was similar to that found with non-pregnant rats at 10^{7}-10^{8} CFU/g of stool. Gestation of the two control animals continued normally with live births of eleven neonates each at E20 and E21. No neonatal mortality within and beyond the seven day p*ost-partum* period was found. Two of A192PP-colonized animals gave birth to live offspring, one littering five and the other eight neonates. There was a rapid onset of mortality of these neonates with no survival at P3. *E. coli* K1 was found in the blood compartment and brain tissues of these animals. Differences in mortality between the offspring of colonized and control animals illustrated vertical transmission of the pathogen and neonatal systemic infection. Interestingly, two colonized adults did not produce live offspring and suffered blood loss at E17 and E18, indicative of spontaneous abortion.

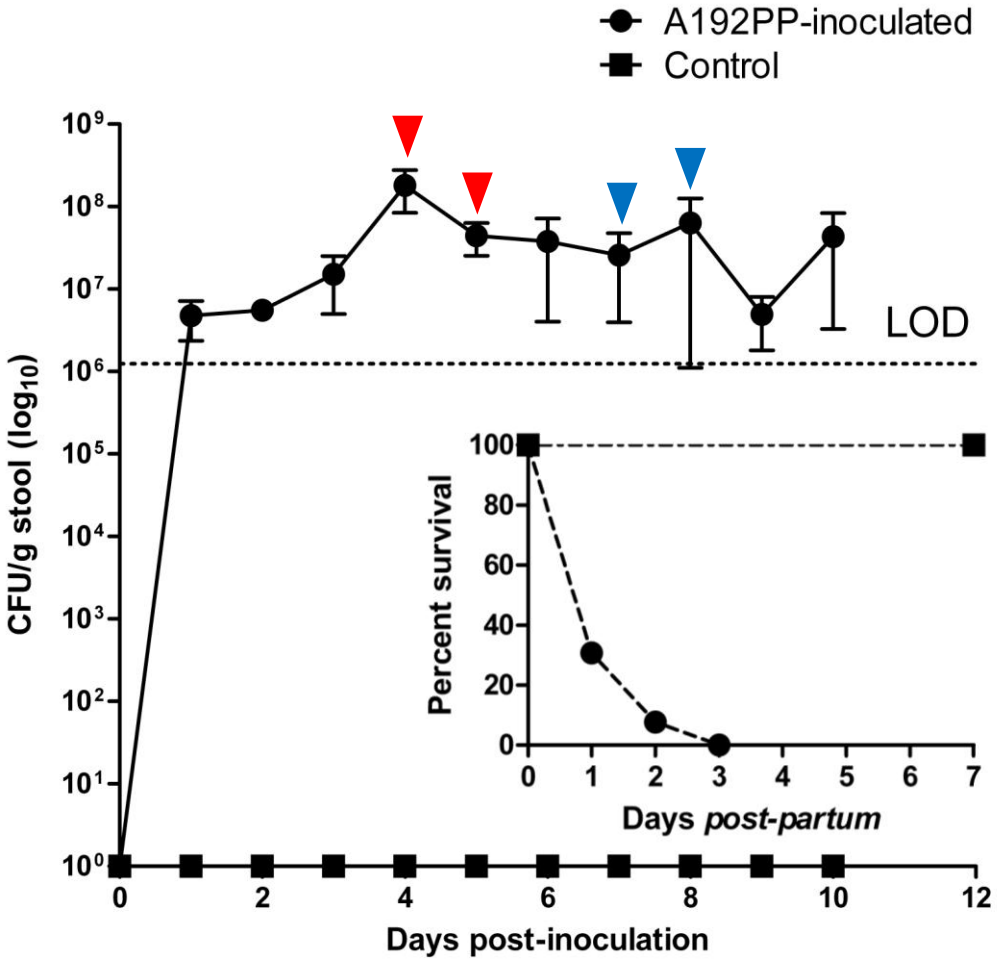


Figure 2.7: *Colonization of pregnant rats with E. coli K1 and transmission to neonates. Four pregnant rats were inoculated with 10^{10} CFU A192PP and two were inoculated with MH broth (control); E. coli K1 was quantified from stool samples. Pregnant rats either aborted (▼) or gave birth (▼) to offspring. Neonatal survival was monitored post-partum (inset). Error bars represent the SEM of CFU/g of stool from four rats. LOD; limit of detection.*

2.3.3 Quantification of *E. coli* K1 by *neuS* qPCR

Culture methods for detecting *E. coli* K1 in stool and tissue samples are laborious and relatively insenstivie; a qPCR assay based on the *neuS* gene of the K1-capsule biosynthesis and export *kps* gene cluster was therefore developed. The *neuS* gene was selected as a single copy gene (*neuS* copy number equals *E. coli* K1 cell number) of the restricted region II of the *kps* cluster with no DNA sequence homology

to any currently deposited DNA sequences, excluding *E. coli* K1 sequences, as determined by BLAST analysis (http://blast.ncbi.nlm.nih.gov). The primer pair NeuSF3 and NeuSR3 were determined by Primer-BLAST to be *neuS*-specific and were designed to be relatively long (30 bp) with a high annealing temperature (60 °C) to increase the stringency of the PCR and to compensate for any partial homology with unrecorded DNA sequences present in the intestinal microbiota.

2.3.3.1 Specificity of the primers

The specificity of the NeuSF3/NeuSR3 primer pair was tested *in vitro* against a range of Gram-negative genomic DNA. Genomic DNA was extracted from the strains in Table 2.1 and the 332 bp *neuS* fragment amplified by PCR. Amplicons were resolved by agarose gel electrophoresis (Figure 2.8). A single band corresponding to approximately 0.3 kbp was produced by all PCR with *E. coli* K1 strain DNA as a template. Amplification was dependent on the presence of *E. coli* K1 genomic DNA, as reactions utilizing DNA from non- *E. coli* bacterial species and *E. coli* strains other than *E. coli* K1, including the related group II capsular serotype K5 strains, were uniformly negative. Amplification of a band of expected size from the K-12/K1 hybrid strain EV36, but not the K-12 wildtype strain CGSC 5073, lent further support in favour of amplification specificity towards the K1-biosynthesis/export *kps* gene cluster. The identity of the amplicon as a fragment of the *neuS* gene was confirmed by *cleanup* of PCR reactions and DNA sequencing.

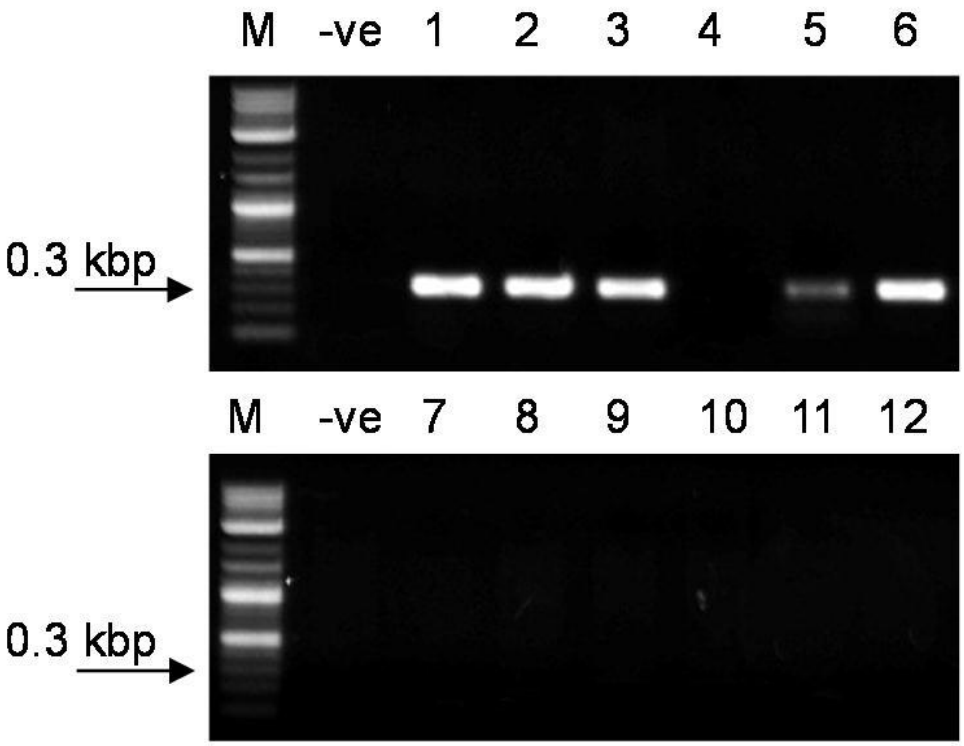


Figure 2.8: *Agarose gel electrophoresis of amplicons produced by neuS PCR using different gDNA templates. Template DNA from strains A192PP (1), C14 (2), DSM 10723 (3), CGSC 5073 (4), EV36 (5), LP1674 (6), LP1395 (7), DSM 10797 (8), DSM 10794 (9), Klspp10 (10), Citro14 (11) and Prmirab42 (12) were used. Lanes containing 2-log ladder DNA (New England Biosciences) and PCR reactions with no template DNA (-ve) are also shown.*

2.3.3.2 Validation of the qPCR assay

The utility of *neuS* qPCR for the quantification of *E. coli* K1 was examined by real-time monitoring of PCR using *E. coli* K1 DNA as a genomic standard and by generation of reproducible standard curves. The use of the technique in quantifying *E. coli* K1 from intestinal tissue and stool homogenates was validated by spiking samples with known quantities of live *E. coli* K1 prior to DNA extraction.

Total genomic DNA was extracted three times from standardized cultures of A192PP; DNA extracts were diluted to 2 x 10^6 gDNA/µL and serially diluted to produce a range of gDNA dilutions for use as qPCR standards. As 5 µL of each

dilution was used in each PCR, this range covered genomic DNA corresponding to 10^1-10^7 CFU A192PP. Representative data produced by real-time monitoring of PCR reactions utilizing these standards as template DNA is shown in Figure 2.9. Amplification of PCR products was detected in all dilutions tested but not in no-template controls (Figure 2.9 A), demonstrating that this method was extremely sensitive and capable of detecting ≤ 10 genome copies. Melt-curve analysis detected a single PCR product with an estimated Tm of 78 °C (Figure 2.9 B) which approximates the Tm of 77.88 °C calculated for the amplified *neuS* fragment sequence. The Ct values produced by amplification of standard DNA from replicate cultures were highly reproducible, allowed the generation of standard curves (Figure 2.9 C) and the determination of PCR efficiencies, which ranged from 96-102%. Thus, *neuS* PCR falls within the parameters required for accurate qPCR-based quantification and represents a valid method for quantification of *E. coli* K1.

Sample spiking was used to determine the capacity of the qPCR assay to quantify *E. coli* K1 DNA from samples containing complex mixtures of bacterial and host DNA. DNA was extracted from four adult stools and neonatal tissue homogenates containing no *E. coli* K1 detected by culture and phage typing. PCRs containing these DNA extracts were spiked with known quantities of A192PP DNA representing a range of 10^1-10^6 CFU. *E. coli* K1 was quantified by *neuS* qPCR and the results compared to spiked CFU values (Figure 2.10). Within the 10^1-10^5 CFU spike range, no significant differences were observed between spike inoculum CFU values and qPCR results derived from analysis of DNA extracted from either stool or tissue homogenates.

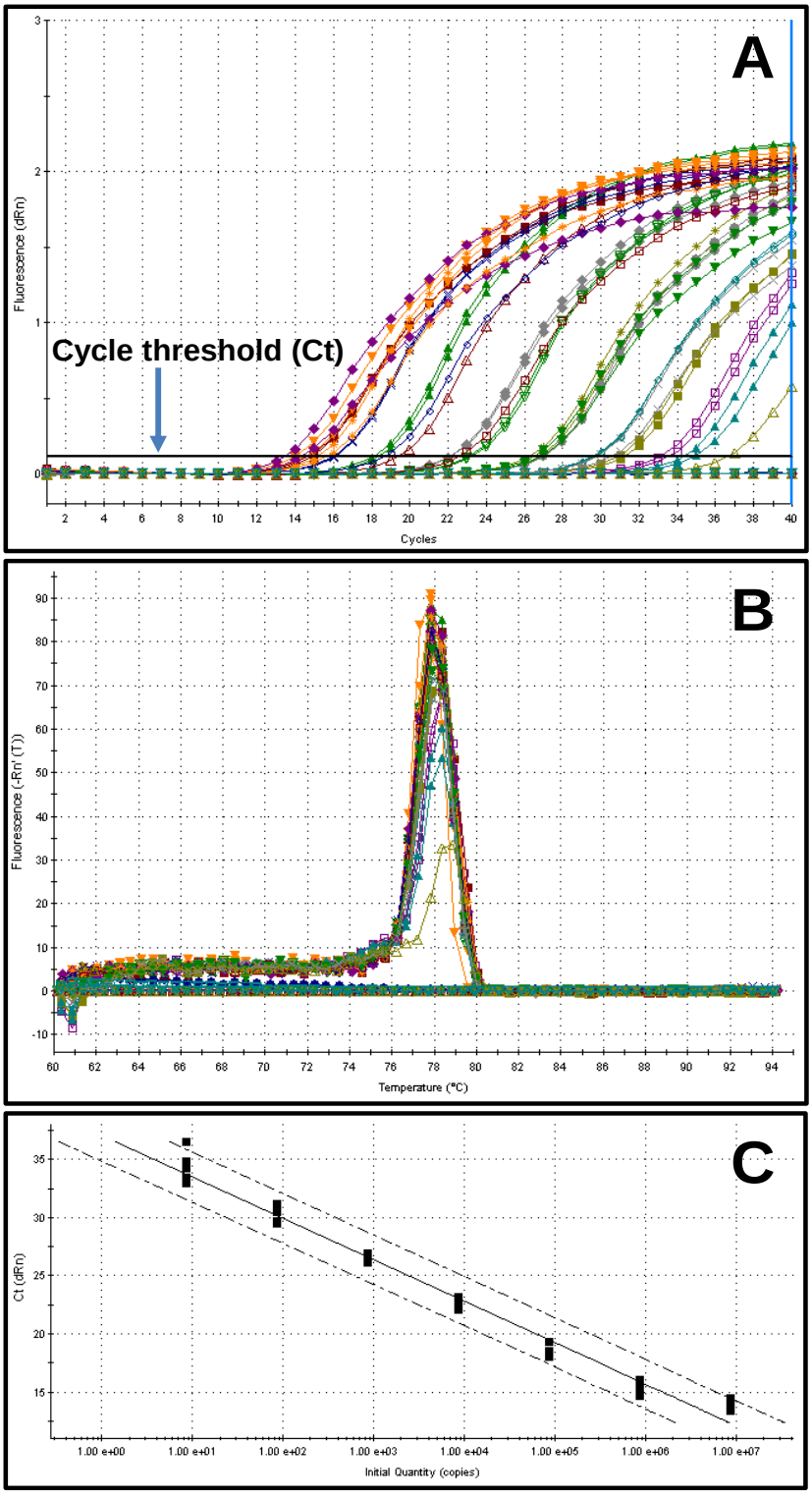


Figure 2.9: *qPCR of the neuS gene using tenfold serial dilutions of A192PP gDNA. DNA was extracted from A192PP. Quantities of DNA corresponding to 10^1-10^7 CFU were amplified by PCR and reactions monitored in real-time. (A) PCR cycle number against fluorescence. Post-amplification reactions were subjected to melt-curve analysis (B), comparing temperature and Δ(fluorescence). Standard curves (C) were constructed by plotting copy number (CFU) against Ct values obtained in A. The cycle threshold is indicated in A and the 95% confidence interval (----) in C.*

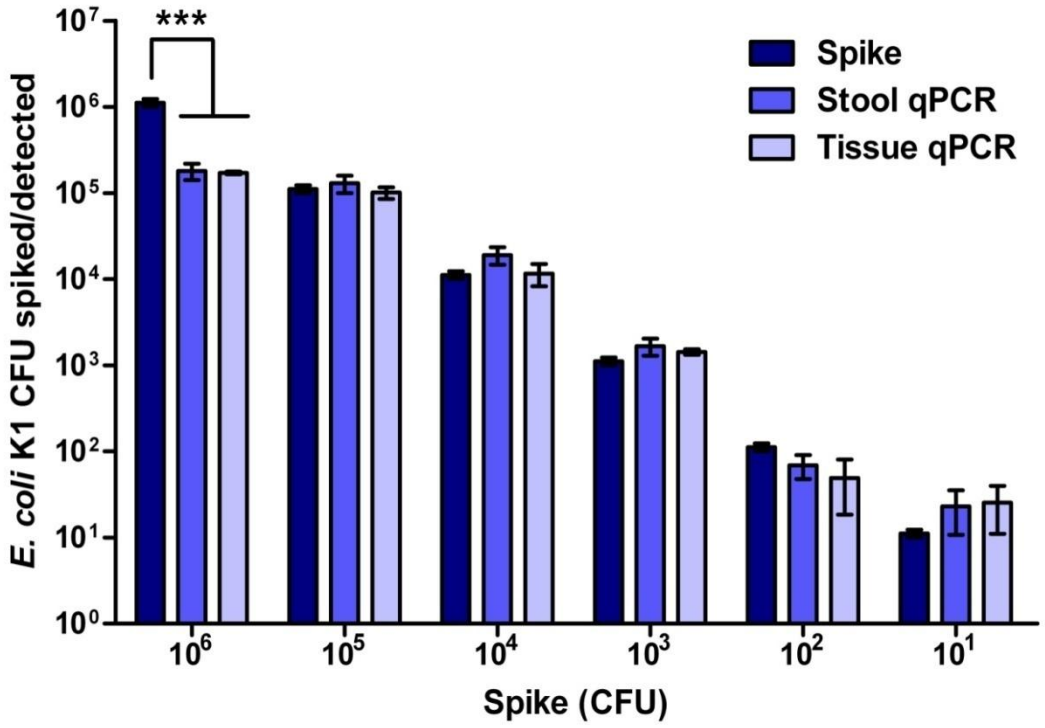


Figure 2.10: *E. coli K1 detected by qPCR of DNA extracted from adult stool and neonatal tissue homogenates spiked with known quantities of A192PP DNA. Error bars represent the SEM from four independent experiments. Differences determined by two-tailed t-test are indicated (*** $p<0.001$).*

However, significantly less *E. coli* K1 was detected by qPCR in both sample types in assays utilizing a 10^6 CFU spike. Melt-curve analysis of PCRs from spiked samples indicated a single amplification product with the same Tm observed previously (Figure 2.9 B). No amplification was observed in non-spiked stool and tissue samples. These results demonstrate the capacity of the *neuS* qPCR assay to quantify *E. coli* K1 DNA from adult and neonatal intestinal DNA extracts and shows that the upper limit of detection of the assay is approximately 10^5 CFU for each PCR.

2.3.3.3 Comparison of culture/phage and qPCR methods *in vivo*

The capacity of the *neuS* qPCR method to quantify *E. coli* K1 from animal samples was compared to 'gold-standard' culture and phage typing. DNA was extracted from the intestinal tissue homogenates of 24 P2 neonatal pups and stool homogenates from twelve A192PP-colonized adult rats and qPCR compared to culture and phage-typing methods (Figure 2.11). Two sub-populations were resolved. With the majority of samples, there was a significant correlation between culture/phage and qPCR data for both tissue (n=21) and stool (n=8), with Spearman R^2 values of 0.87 and 0.95 respectively. However, a minority of tissue (n=4) and stool (n=4) samples yielded *E. coli* K1 by qPCR but not by culture/phage typing. Melt-curve analysis of DNA amplified from these samples indicated a single product with the same Tm as the *neuS* amplification product. Moreover, the CFU/g values determined by qPCR were either near or below the LOD for culture and phage typing, as determined previously by normalization to mean tissue and stool mass, indicating that qPCR detected *E .coli* K1 from samples that were negative by culture/phage typing. Calculation of qPCR LOD values for both sample types, based on the dilution steps required for DNA extraction and the sensitivity of the qPCR assay, showed that qPCR was 62.5-fold more sensitive for quantification of *E. coli* K1 than culture/phage typing. Taken as a whole, these results demonstrate that quantification of *E. coli* K1 by qPCR assay was more sensitive and more reliable than culture and phage typing.

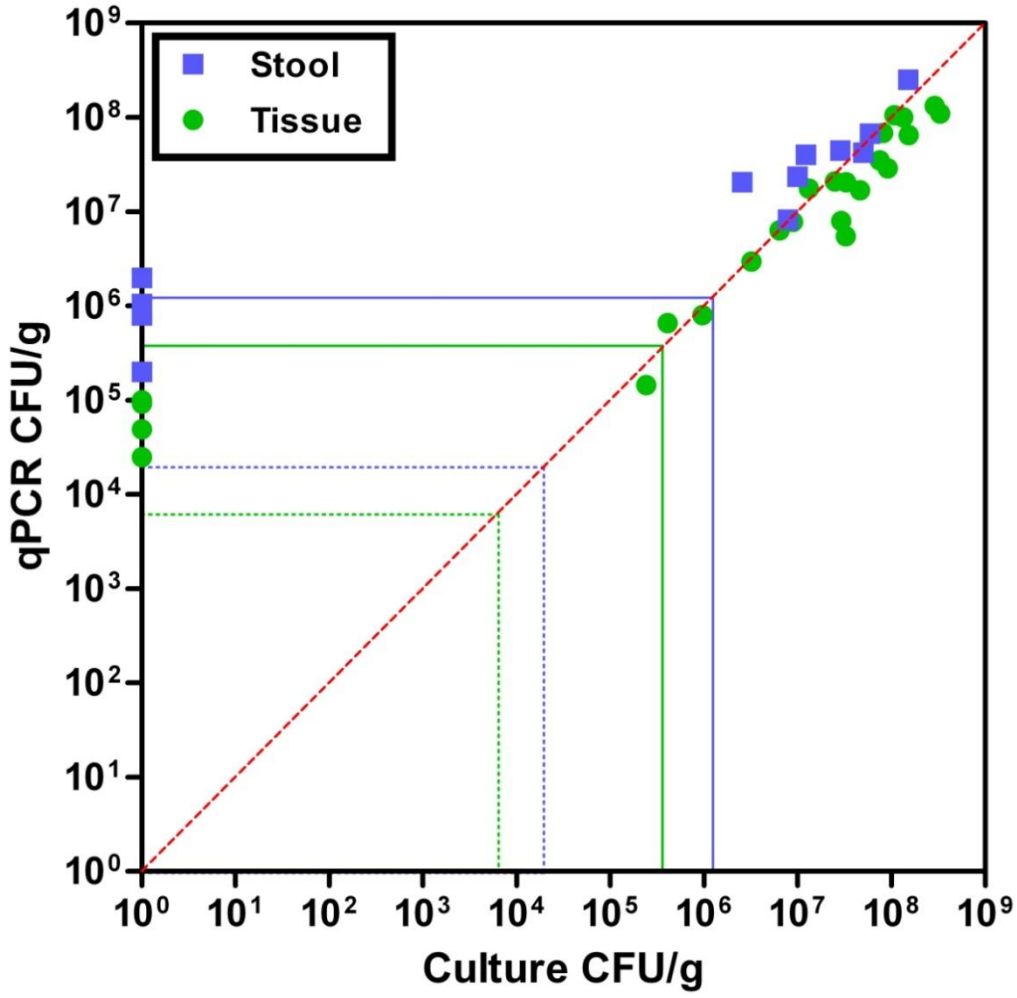


Figure 2.11: *Comparison of E. coli K1 CFU/g detected by qPCR and culture methods. E. coli K1 was quantified from 24 neonatal tissue and twelve adult stool homogenates from A192PP-colonized animals. The LODs of culture (solid lines) and qPCR (dotted lines) are indicated for stool (blue) and tissue (green) samples.* R^2 *=1 (perfect correlation; ----).*

2.4 Discussion

The neonatal rat model of *E. coli* K1 infection has been used for almost forty years to investigate the pathogenic mechanisms which drive the infectious process (Kim *et al.*, 1992; Sukumaran *et al.*, 2003; Zelmer *et al.*, 2008) and to examine the efficacy of novel biotherapeutic measures to promote clearance of the pathogen from the neonatal circulation (Mushtaq *et al.*, 2005; Zelmer *et al.*, 2010). Here, I have further characterized the model in relation to the age dependency of systemic infection and mortality in neonatal rats colonized with a highly virulent *E. coli* K1 strain.

In our version of the model, the majority of neonates become refractive to systemic disease at approximately P7, with no mortality observed in any P9 animals. The only other publication to analyse the survival of neonates dosed at different time-points *post-partum* (Glode *et al.*, 1977) examined the survival of rats inoculated at P3, P5, P15 and P30 and found a relatively low mortality rate (6-8%) in all age groups apart from P30. Although this study indicates age dependency of systemic infection and mortality it differs from that reported here; these differences may be method-dependent. The challenge inoculum of Glode *et al.* was substantially higher at 10^8-10^{10} CFU compared to 10^7 CFU in the present study. Furthermore, the strain utilized by Glode *et al.* (C94) did not appear to colonize the GI tract of the rat particularly well, with colonization reported to be as low as 19% five days after inoculation, whereas the A192PP strain used in this study had colonized all members of all age groups by 72 h. Although a relatively high proportion of P3 and P5 C94-colonized neonates developed bacteraemia, only a small fraction of these animals developed lethal meningitis, in contrast to the present study with A192PP. These differences make a comparison of the two studies difficult. However, the use of a fixed inoculum size and a strain with a high colonization rate and strong bacteraemia/mortality relationship minimized these potential sources of variation and clarified temporal issues relating to development of resistance to infection.

The strong correlation between bacteraemia and mortality in susceptible neonates but not in older resistant cohorts provided further evidence that the capacity of the pathogen to translocate from the intestines into the bloodstream is a primary factor

in the determination of the host's susceptibility to systemic infection. Resistance to systemic infection began to emerge during the P4-P6 period, as evidenced by the intermediate susceptiblity to systemic disease of animals within this age group. Examination of phage-mediated clearance of *E. coli* K1 from the intestines of P2-colonized neonates provided evidence that the pathogen enters the systemic circulation within 24-72 h of colonization and may account for the partial susceptibility to infection of the intermediate-susceptible group. Thus, P2 and P9 neonates represent *E. coli* K1-susceptible and refractive phenotypes and pups of these age groups will be used for further investigations of age dependency.

The attempt to develop an infection model of maternal-neonatal pathogen transmission showed that orally-induced, stable intestinal colonization of the pregnant adult rat was feasible but had some drawbacks. Intestinal colonization of the pregnant rat by *E. coli* K1 had a severely detrimental effect on both the gestation process of the foetus, as evidenced by spontaneous abortion and small litter size of colonized animals and the poor survival prospects of neonates. The most likely explanation for these effects is that the genital tract of colonized pregnant rats was contaminated with A192PP shed from the intestines; these bacteria may have ascended to the uterus and infected the developing foetus. *In utero* infections in the rat have been shown previously to result in loss of the foetus and/or poor survival rates (Payne, 1960) and could account for the effects observed in this study. Whilst vertical transmission of the pathogen was replicated in this model, the paucity in the number of offspring and the rapidity with which they are lost limit the use of this procedure with respect to *E .coli* K1 pathogenesis. However, the model could prove useful in future investigations of *E. coli* K1 prophylaxis by clearance of the pathogen from maternal reservoirs of infection, or in the investigation of *E. coli* K1 *in utero* infections.

The development of a qPCR-based assay for quantification of *E. coli* K1 from GI samples by determination of *neuS* gene copy number is reported here. The primers utilized to target the gene were specific and capable of amplifying the gene from mixed GI-extracted DNA samples in both DNA spike assays and samples from the colonized rat. The use of qPCR to quantify different bacterial groups and species, including *E. coli*, from heavily contaminated environments is not novel. *E. coli* has been quantified from GI mucosal (Huijsdens *et al.*, 2002) and environmental samples (Khan *et al.*, 2007). These authors utilized a set of targets that included the 16S and 23S rRNA

subunit genes and the internal transcribed spacer (ITS) region which sits between these structural RNA genes. The conserved and variable regions of bacterial rRNA genes make them useful targets for these assays; however they are not suitable for differentiating *E. coli* pathovars that do not possess a useful degree of ribosomal genetic diversity. Thus, the targeting of pathovar-specific genes such as *neuS* represents a more viable alternative. Although the use of *neuS* PCR to detect K1 antigen has been previously investigated (Tsukamoto, 1997) it has not been used as a means of quantification prior to this report. A drawback to utilizing qPCR to quantify bacteria is that the method makes no distinction between DNA extracted from live cells and DNA fragments expelled during lytic cell death. However, studies indicate that the survival of intact naked DNA in the GI tract of both rats and humans is extremely transient, most likely due to the high expression of secreted DNase by intestinal tissues and hydrolysis by intestinal microbiota (Lacks, 1981; Maturin & Curtis, 1977; Netherwood *et al.*, 2004; Schubbert *et al.*, 1994). The strong correlation between qPCR and culture/phage-typing is supportive of this assertion and validates the qPCR method; however, it should be noted that extra-intestinal sites may lack this degradative capacity and may not be suitable for use in conjunction with this technique.

CHAPTER 3

THE INTESTINAL MICROBIOTA

3.1 Introduction

The intestinal microbiota plays an essential role in both the stimulation of intestinal development and the provision of an enhanced metabolic capacity to the host (Stappenbeck *et al.*, 2002; Cebra, 1999; Round & Mazmanian, 2009; Flint *et al.*, 2007; Bäckhed *et al.*, 2004; Resta, 2009). The protective function of the microbiota is also of significant importance in preventing infection by opportunistic pathogens. This protective function is a component of a mechanism designated colonization resistance (CR), defined as the growth restriction and/or clearance of exogenous or indigenous pathogens from the GI tract. The other component of CR is the host tissues (reviewed by Vollaard & Clasener, 1994; Stecher & Hardt, 2010). It is useful to separate CR into microbiota-mediated CR (mCR) and host-mediated CR (hCR) mechanisms. hCR is mediated by the physiology of the GI tract (for example, gastric acid, bile salts and intestinal motility) and by innate and adaptive elements of the intestinal immune system characterized by AMP and secretory IgA (sIgA) production respectively. mCR is based on at least three different mechanisms: direct inhibition, competitive inhibition and the stimulation of hCR mechanisms.

Direct inhibition of colonization is mediated by the production of molecules which are toxic to the incoming pathogen. These molecules include metabolites such as acetate and short-chain fatty acids like butyrate which have an inhibitory effect on the growth of some pathogenic bacteria (Hopkins & Macfarlane, 2003). Many bacteria also secrete a range of narrow spectrum antibiotics, the bacteriocins, which predominantly target closely-related bacteria (Rea *et al.*, 2010) but can also have broader spectrum activities (McAuliffe *et al.*, 1998; Rea *et al.*, 2007). Competitive inhibition is based on the denial of vital nutrients and mucus receptor binding sites to the pathogen by the endogenous flora. The high bacterial load in the intestines (~10^{12} microbes/mL) means that space available for pathogen binding, preventing removal from the lumen by flushing mechanisms, is severely limited. Nutrients are relatively scarce in the enteric environment, as most are absorbed by intestinal enterocytes and the commensal microbiota has evolved to efficiently utilize the remainder. This leaves very little for the incoming pathogens to exploit as a metabolic basis for growth (reviewed by Stecher & Hardt, 2008).

The microbiota is also vital in mediating clearance of pathogens from the intestinal lumen and preventing colonization. Pathogenic members of the Enterobacteriaceae, such as *Salmonella* spp, can disrupt the ecology of the microbiota by provoking host inflammatory responses in the intestine (Lupp *et al.*, 2007; Stecher *et al.*, 2007). Such inflammatory responses cause alterations in the composition of the microbiota, which reduces the efficacy of mCR mechanisms. Restoration of the 'normal' microbiota induces clearance of the pathogen from the GI tract in a process which appears to be independent of any known hCR mechanism (Endt *et al.*, 2010).

The microbiota stimulates both innate and adaptive elements of hCR mechanisms. Many hCR mechanisms are constitutively expressed and do not require stimulation by the microbiota, as demonstrated by comparison of germ-free and conventionally reared animals (Putsep *et al.*, 2000; Karlsson *et al.*, 2008). However, elements of the microbiota are required to stimulate production and secretion of components of the innate intestinal defences, including REG3 C-type lectins and angiogenins (Hooper *et al.*, 2003; Vaishnava *et al.*, 2011). The microbiota is sampled by intestinal dendritic cells, which then induce the production of sIgA in intestinal B-cells (Macpherson & Uhr, 2004), an event which does not occur in germ-free animals (Bevenis *et al.*, 1971). sIgA functions as both a neutralizing agent and immunological activator (reviewed by Corthesy, 2007) and the diversity of the sIgA repertoire of the intestine increases substantially with age (Lindner *et al.*, 2012). Neonates are therefore deficient in this adaptive element of intestinal defences but ingestion of sIgA, acquired as a component of maternal breast milk (Hanson, 1999), compensates for this deficiency.

The relationship between *E. coli* K1 and the intestinal microbiota is not well characterized. However, probiotic species of *Lactobacillus* reduce *E. coli* K1 binding to, and translocation across, epithelial cells *in vitro* and they can prevent haematogenous dissemination of the pathogen from the rat intestine (Huang *et al.*, 2009; Lee *et al.*, 2000). Lactobacilli are prevalent in the maternal vaginal microbiota and as such are one of the first microbes encountered by the neonate, forming a consistent component of the neonatal pioneer GI microbiota (Karlsson *et al.*, 2011). The exact mechanism by which this Gram-positive genus provides protection against *E. coli* K1 remains unclear, although there is evidence that it induces mucin expression in colonic epithelial cells

(Dykstra *et al.*, 2011) that can prevent binding of EPEC and EHEC strains to the epithelial cell surface (Mack *et al.*, 1998).

mCR is important for the maintainance of host intestinal tissue defences. It can be hypothesised that the dynamic state of the neonatal intestinal microbiota immediately *post-partum* (Palmer *et al.*, 2007) influences the capacity of *E. coli* K1 to translocate from the intestinal tract. The landmark study by Palmer *et al.* established that the neonatal microbiota is dominated by Gammaproteobacteria and certain classes of the phylum Firmicutes. The phylum Bacteroidetes is initially absent or transiently present but their number increases over the first year of life as the microbiota matures towards the adult phenotype. This study also demonstrated that the bacterial load in the human intestines varies substantially in the first week *post-partum*, starting from a relatively low point and increasing and stabilizing between P5-P10. If the microbiota is a key factor in determining susceptibility to *E. coli* K1, then the quantitative and qualitative dynamism of the neonatal microbiota could play a role in the determination of systemic infection (Figure 3.1).

Analysis of complex microbial communities has benefitted from the advent of modern molecular methods. A common basis of such analyses is the gene coding for the 16S rRNA, which forms the structural scaffold of the 30S (small) subunit of the prokaryotic ribosome. This gene is designated as small-subunit ribosomal DNA (SSU rDNA) and is a component of the multi-copy *rrn* operon. The SSU rDNA sequence is a mosaic of highly conserved and hypervariable regions (Figure 3.2). These features have made SSU rDNA the target of choice for examination of the phylogenetic relationships between prokaryotic lineages (O'Neill *et al.*, 1992). Multiple metagenomic techniques have evolved to enable the characterization of complex microbial communities based on the quantification of specific SSU rDNA sequences. These include quantitative microarray-based analysis and direct sequencing methods. The advent of high-throughput DNA sequencing technologies means that sequencing is now considered the method of choice for microbial community analysis (Gill *et al.*, 2006), but the use of this technology is still restricted by the high cost of the sequencing platforms. DNA microarrays consisting of probes which target the hypervariable regions of the SSU rDNA sequence present a viable alternative to direct sequencing and use pre-

Figure 3.1: *The potential role of the quantitative (A) or qualitative (B) dynamism of the neonatal microbiota in determining susceptibility to E. coli K1 infection.*

Figure 3.2: *The 1.5 kb SSU rDNA sequence. Highly conserved (C; blue) and hypervariable (V; green) regions are highlighted.*

existing microarray processing infrastructure. One such array was designed by Palmer *et al.* (2008) and employs probes corresponding to SSU rDNA sequences of 649 of the 950 taxonomic groups in the prokaryotic multiple sequence alignment (prokMSA) database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). The microarray incorporates species-specific probes for 1,590 bacterial and 39 archeal species, ensuring that 94% of the ~16,000 operational taxonomic units (OTUs) in prokMSA are represented at least once at some taxonomic level.

Another useful tool to study the impact of the microbiota is the axenic or 'germ-free' (GF) animal model. GF animals are born and raised in sterile conditions, do not possess any element of the normal microbiota and thus function as microbiota knockout models. GF animals consistently exhibit increased susceptibility to infection mediated by a variety of pathogens in enteric infection models (Inagaki *et al.*, 1996; Nardi *et al.*, 1989; Tazume *et al.*, 1990). However, data from GF infection models must be interpreted with caution, for two reasons. Firstly, they do not allow differentiation between different mCR mechanisms. Secondly, colonization by the microbiota triggers host developmental pathways beyond the stimulation of hCR mechanisms and as a consequence, the environment encountered by the pathogen in GF infection models may not be representative of the natural setting of disease (reviewed by Sekirov *et al.*, 2010). A means of circumventing these disadvantages is by suppression of the microbiota in conventionally reared animals. This provides an experimental host which has received the normal developmental stimuli provided by enteric colonization, but which has a reduced intestinal microbiota. Such suppression can be achieved experimentally by the use of combined antibiotic treatment (Membrez *et al.*, 2008; Croswell *et al.*, 2009).

This chapter describes experiments designed to clarify the role of the intestinal microbiota in the determination of susceptibility to *E. coli* K1 infection in the neonatal rat. I have therefore undertaken analysis of the colonization kinetics of *E. coli* K1 in susceptible and refractive neonates, quantitative and qualitative profiling of the neonatal microbiota of neonates of the different susceptibility groups and an assessment of the impact of antibiotic-mediated suppression of the microbiota on susceptibility to *E. coli* K1.

3.2 Methods & Materials

Unless otherwise indicated, growth media were purchased from Oxoid Ltd and chemicals, reagents and enzymes from Sigma-Aldrich. Oligonucleotides were synthesised by, and purchased from, Eurofins MWG Operon. All reagents used in microarray sample preparation and hybridizations described in sections 3.2.5 and 3.2.6 were purchased from Agilent Technologies. SSU rDNA analytical methods are broadly based on methods described by Palmer *et al.* (2008). This section describes methods which are specific to the results described in this chapter; however, some methods used in Chapter 2 were also employed.

3.2.1 SSU rDNA PCR primers

A number of primers were used; primer sequences, target regions on the SSU rDNA sequence and original source references are shown in Table 3.1.

Primer	Sequence (5'-3')	Target	Reference
8FB	AGGGTTCGATTCTGGCTCAG	C1	Palmer *et al.*, 2008
Bact515R	TTACCGCGGCKGCTGGCAC	C3	Lane *et al.*, 1985
8FM	AGAGTTTGATCCTGGCTCAG	C1	Lane *et al.*, 1985
1391R	GACGGGCGGTGTGTRCA	C8	Lane *et al.*, 1985

Table 3.1: *Sequences, conserved SSU rDNA target regions and source references of primers used in SSU rDNA PCR experiments.*

3.2.2 SSU rDNA qPCR

SSU rDNA copy numbers in tissue and stool samples were quantified by qPCR.. DNA was extracted from *E. coli* K-12 strain CGSC 5073 (see section 2.2.8). Genomic standards with known SSU rDNA copies/µL values were prepared by tenfold serial dilution. Standards used in qPCR typically ranged from 2-2000 SSU rDNA copies/µL. qPCR reactions were performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix kits (Agilent Technologies) according to manufacturer's instructions. Universal forward primer 8FM (900 nM), *Bifidobacterium longum* forward primer 8FB (90 nM), universal reverse primer Bact515R (900 nM) and ROX reference dye (30 nM) were added to each qPCR. Reactions were prepared in light-protected tubes and in a C2BSC to reduce the risk of DNA contamination. qPCR mixes (15 µL/reaction) were dispensed into 96-well PCR plates. Genomic standard DNA, experimental sample DNA, or nuclease-free ddH_2O (acting as a no-template control) were added to each qPCR mix to give a final volume of 20 µL per reaction. Wells were sealed using optically clear strip caps. qPCRs were run on an Mx3000P system (v.2 software; Stratagene) set to detect SYBR1 and ROX fluorescence, utilizing a thermal cycling program comprising 95 °C for 3 min, 40 cycles of 95 °C for 20 s, 55 °C for 20 s, 60 °C for 35 s, 65 °C for 15 s and 72 °C for 15 s. Fluorescence was measured at the 72 °C step of each amplification cycle and amplification curves recorded. DNA melt curves were generated by cooling reactions to 55 °C and increasing the temperature to 95 °C over 30 min with fluorescence measured every 20 s. SYBR1 fluorescence was normalized to ROX fluorescence and the SYBR1 amplification curves were used by the software to generate Ct values utilizing adaptive baseline and amplification-based threshold algorithm enhancements. Genomic standard Ct values were used to generate standard curves for the calculation of sample SSU rDNA copies/µL and these values were normalised to original sample (tissue/stool) mass. Each qPCR reaction plate utilized standard DNA extracted from three separate CGSC 5073 cultures and each standard, sample and control reaction was duplicated on each plate. Each plate was replicated and data for each sample averaged across the four replicate values.

3.2.3 Whole SSU rDNA amplification and cleanup

Purified whole SSU rDNA was prepared by PCR amplification and cleanup prior to microarray analysis. DNA was extracted and quantified from tissue and stool samples as previously described (section 2.2.9). PCR reactions were performed using GoTaq Green Master Mix (Promega) according to manufacturer's instructions. Universal forward primer 8FM (0.4 µM), universal reverse primer 1391R (0.4 µM) and sample DNA (1 µg) were added to each PCR. PCRs were performed in a Techne Thermocycler (Bibby Scientific). The thermocycling programme comprised 95 °C for 5 min and 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 8 min. Reactions were cleaned using a Wizard SV Gel and PCR Clean-up kit (Promega) according to manufacturer's instructions (section 2.2.11). DNA concentration and purity was assessed using a NanoDrop spectrophotometer (Thermo Scientific). Samples were stored at -20 °C. The presence of a single 1400 bp DNA product was checked by agarose electrophoretic resolution by mixing 10 µL of DNA with 2 µL of 6 x Gel Loading Buffer, loading the mixture onto a 1% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide and performing electrophoresis at 80 V in Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate, 1mM EDTA, pH8) for 30 min or until the dye front reached the end of the gel. DNA was visualized by scanning the gel with a Molecular Imager FX system (Bio-Rad) set to detect UV fluorescence.

3.2.4 Microarray reference pool

A reference pool of SSU rDNA amplicons was constructed for use in subsequent microarray co-hybridizations with experimental sample amplicons according to the Palmer *et al.* (2008). This served as a common reference to allow data normalization between microarrays and served to increase the stringency of the microarray hybridizations by competing with experimental sample amplicons for binding to microarray probes. The reference pool comprised an equimolar mixture of cleaned SSU rDNA amplicons from all experimental (108 tissue and 80 stool sample) DNA extractions.

3.2.5 SSU rDNA amplicon labelling and purification

Reference pool and experimental SSU rDNA amplicons were fluorescently labelled with, respectively, Cy3 or Cy5 dye-conjugated nucleotides using Genomic DNA Labelling Kit Plus reagents according to manufacturer's instructions. The compositions of buffers utilized in the labelling process were proprietary information unless otherwise indicated. All labelling steps employed light-protected tubes to prevent Cy3 and Cy5 photobleaching. SSU rDNA amplicons were diluted to 19.23 ng/µL in nuclease-free ddH_2O, mixed with 5 µL of random primers, heated to 95 °C for 3 min and incubated on ice for 5 min. Batches of Cy3 and Cy5 labelling master mix were prepared (containing Cy3/Cy5-dUTP; 60µM) and mixed with amplicons to a final volume of 50 µL for each labelling reaction. Mixtures were incubated at 37 °C for 2 h and 65 °C for 10 min. Individual Cy5-labelled experimental SSU rDNA amplicons were mixed with an equal volume (50 µL) of Cy3-labelled reference pool SSU rDNA amplicons. Labelled amplicon mixtures were purified using MinElute DNA Cleanup Kits (Qiagen) according to manufacturer's instructions. Reactions were mixed with 500 µL of Buffer PB and applied to spin-column membranes. Columns were centrifuged at 13000 x *g* for 1 min and the filtrate discarded. Columns were washed twice with Buffer PE (500/250 µL) and centrifuged at 13000 x *g* for 1 min after each wash. Amplicons were eluted in 20 µL of nuclease-free water and 2 µL used to confirm dye-incorporation with a NanoDrop spectrophotometer (Thermo Scientific). The remaining 18 µL were used for hybridization to microarray slides.

3.2.6 Microarray hybridization and washing

Labelled SSU rDNA amplicons were hybridised to SSU rDNA Custom cGH microarray slides, using the basic format of Palmer *et al.* (2008), in 4 x 2 array per slide format and were washed prior to scanning. Array hybridizations utilized Oligo aCGH/ChIP-Chip Hybridization Kit reagents. The composition of buffers for hybridization and washing were proprietary information unless otherwise indicated. Blocking Agent and Hybridization Buffer were mixed with labelled SSU rDNA amplicons to a final volume of 45 µL; hybridization mixtures were heated to 95 °C for 3 min and incubated at 37 °C for 30 min. Hybridization chambers were assembled by

dispensing mixtures into 8 x 2 slide gaskets, aligning array slides on top of each gasket slide and clamping the two slides tightly together. Chambers were placed in a rotating hybridization oven (20 rpm) and incubated at 65 °C for 24 h. Hybridization chambers were disassembled in troughs containing Oligo aCGH Wash Buffer 1. Microarray slides were transferred to fresh Oligo aCGH Wash Buffer 1 and incubated at room temperature for 5 min with stirring. Slides were washed in Oligo aCGH Wash Buffer 2 for 1 min at 37 °C with stirring and transferred to acetonitrile for 10 s. Slides were submerged in Stabilization & Drying Solution for 30 s and briefly air dried. Washed array slides were stored in light-protected containers prior to scanning.

3.2.7 Microarray scanning and data normalization

Microarrays were scanned in an Agilent High Resolution C Scanner at a 5 µm resolution with the extended dynamic range setting at 100 & 10. Cy3 and Cy5 dyes were detected using, respectively, 532 nm and 640 nm lasers. Microarray images were processed using Feature Extraction software v. 9.5.1.1 with linear normalization, rank consistent probe dye normalization methods and background signal was corrected by averaging across all negative control array features. Data was processed using GeneSpring GX (v. 7.3.1) to combine data from replicate spots on each array and merge data from replicate arrays by normalization to standard Agilent array control probes and SSU rDNA array-specific positive and negative control probes. Probes were filtered to remove any reporters with normalized Cy5 + Cy3 fluorescence values of $\leq$1000. Combined and normalized Cy5:Cy3 ratios were computed for individual filtered reporters, allowing relative quantification of SSU rDNA sequences between different experimental samples.

3.2.8 Preparation of competent A192PP cells

Competent cells of *E. coli* K1 strain A192PP were prepared prior to transformation with pUC19 plasmid. Single MH agar cultured colonies were used to inoculate 10 mL of LB broth and the tubes incubated overnight at 37°C in an orbital incubator. The overnight culture was used to inoculate 50 mL of sterile LB broth at a

dilution of 1:100 and incubated at 37°C in an orbital incubator until an OD_{600} 0.5 was reached. Bacterial cells were sedimented by centrifugation at 5000 x *g* for 10 min at 4 °C and the supernatant discarded. Cell pellets were suspended in 25 mL of 0.1 M $MgCl_2$ chilled to 4 °C and cell suspensions incubated on ice for 1 h. Bacterial cells were sedimented by centrifugation at 5000 x *g* for 10 min at 4 °C and the supernatant discarded. The cell pellet was suspended in 5 mL of 0.1 M $CaCl_2$ chilled to 4°C and the cell suspension incubated on ice for 30 min. Competent cells were assayed for viability by plating onto MH agar and incubated at 37 °C to check for growth. Cells were mixed with an equal volume of sterile 20% (v/v) glycerol and stored at -80 °C prior to transformation.

3.2.9 Transformation of competent A192PP with pUC19

Competent A192PP cells were transformed with plasmid pUC19. Competent A192PP cells were mixed with 100 ng of pUC19 (New England Bioscience) and incubated on ice for 15 min. Cells were subjected to heat shock at 42 °C for 40 s and returned to ice for 1 min. Cell were mixed with 500 µL of sterile LB broth and incubated at 37 °C for 45 min. Cell suspensions were concentrated by centrifugation at 5000 x *g* for 10 min and suspended in 100 µL of PBS. The suspension was serially diluted tenfold to a factor of 10^{-3}, each dilution plated onto selective agar (ampicillin [100 µg/mL] in MH agar) and incubated overnight at 37 °C. Single transformed colonies were inoculated into 10 mL of sterile MH broth containing ampicillin (100 µg/mL) and grown to OD_{600} 0.5. Transformants were checked for K1 capsule expression by sensitivity to the K1E bacteriophage (section 2.2.7). Transformant cultures were mixed with an equal volume of sterile 20% (v/v) glycerol and stored at -80 °C. A single K1E-sensitive transformant colony was isolated and designated A192PPR.

3.2.10 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) at which antibiotics prevented growth of *E. coli* K1 strains A192PP and A192PPR was determined in order to assess their sensitivity to ampicillin, streptomycin, metronidazole and vancomycin. MIC assays were performed in 96-well format according to the standard microdilution method of the Clinical and Laboratory Standards Institute (CLSI; http://www.clsi.org). Stock solutions of each antibiotic were prepared by dissolving ampicillin sodium salt, streptomycin sulphate, vancomycin HCl hydrate or metronidazole in sterile MH broth to a concentration of 2.56 mg/mL. Antibiotic solutions were sterilized by filtration with 0.22 µm MILLEX GP filters (Millipore) and the filtrate stored at 4 °C. Single MH agar colonies were used to inoculate 10 mL of MH broth and the tubes incubated overnight at 37 °C in an orbital incubator. The overnight culture was used to inoculate 10 mL of MH broth at a dilution of 1:100 and incubated at 37°C until OD_{600} 0.13 (McFarland Standard 0.5) was reached. MIC plates were prepared by twofold serial dilution of antibiotic stock solutions in MH broth in U-shaped 96-well plates (Corning) with 100 µL antibiotic solution per well. Bacterial cultures were diluted to 10^6 CFU/mL in MH broth and 100 µL of bacteria were dispensed to each well containing antibiotic to produce final antibiotic concentrations over the range 1.25-1280 µg/mL. Control wells containing bacteria or antibiotic alone were prepared for each plate; each test or control was prepared in triplicate on each plate. Plates were sealed, incubated at 37 °C for 24 h and assessed visually for bacterial growth. The lowest antibiotic concentration at which no bacterial growth was observed was recorded as the MIC.

3.2.11 Antibiotic treatment of neonatal rats

Combinations of ampicillin, streptomycin, vancomycin and metronidazole were administered orally to neonatal rats. These antibiotics have been used by previous investigators, either individually or in combination, to suppress the intestinal microbiota (Croswell *et al.*, 2009; Barthel *et al.*, 2003; Rakoff-Nahoum *et al.*, 2004); they represent a broad range of antibiotic classes and antibacterial activity spectra (Table 3.2).

Antibiotic	Class	Mechanism of action	Activity-Spectrum
ampicillin	β-lactam	Cell wall synthesis inhibitor	Broad-spectrum
streptomycin	Aminoglycoside	Protein synthesis inhibitor	Broad-spectrum
vancomycin	Glycopeptide	Cell wall synthesis inhibitor	Gram-positive bacteria
metronidazole	Nitroimidazole	Reduced to genotoxic intermediary*	Anaerobic bacteria

Table 3.2: *Antibiotics used for suppression of the intestinal microbiota. * The mechanism of metronidazole activity is poorly characterized.*

Antibiotic solutions were prepared by dissolving each antibiotic in water; ampicillin and vancomycin were prepared at 400 mg/mL, streptomycin at 120 mg/mL and metronidazole at 10 mg/mL. The solutions were sterilized using 0.22 µm MILLEX GP filters (Millipore) and filtrates stored at 4 °C. Antibiotics were administered to neonatal rats by the oral route using a micropipette. Antibiotics were administered in the same order (ampicillin, streptomycin, vancomycin, metronidazole) on each day of treatment. Dosing volumes were 25 µL for ampicillin, streptomycin and vancomycin and 30 µL for metronidazole, representing a total dose of 10 mg for ampicillin and vancomycin, 3 mg for streptomycin and 0.3 mg for metronidazole. Although there are no known antagonistic interactions between these antibiotics, they were administered individually to neonates, with 1 h between each individual dose. Neonates undergoing antibiotic treatment and concurrent inoculation with *E. coli* K1 strains were inoculated with bacteria 4 h after the last antibiotic dose. Oral inoculation with *E. coli* K1 was performed as described in section 2.2.4.

3.3 Results

3.3.1 *E. coli* K1 intestinal colonization

Temporal aspects of *E. coli* K1 colonization of the neonatal intestine were investigated to shed light on the role of CR and pathogen clearance in the modulation of susceptibility to systemic *E. coli* K1 infection. P2, P5 and P9 neonates were selected as representatives of the three different susceptibility phenotypes (susceptible, intermediate susceptible and refractive) identified in Chapter 2. Three litters of fourteen neonates from each age group were inoculated with mid-exponential phase A192PP and one litter of fourteen neonates from each age-group inoculated with sterile MH broth as negative control. At time-points ranging from 0 h (pre-dose controls) to 120 h after inoculation, two neonates were culled from each litter (six *E. coli* K1-colonized and two controls for each age group and time point. A large proportion of pups in two litters colonized at P2 died and further litters were employed to ensure sufficient live neonates at 96 and 120 h were available.

Intestinal tissues (duodenum to rectum) were removed from culled neonates and the *E. coli* K1 burden determined over a 120 h period by *neuS* qPCR (Figure 3.2). There were no significant differences, at any time point, in the number of *E. coli* K1 between the groups of P2, P5 and P9 neonates ($p > 0.32$; Kruskal-Wallis). The *E. coli* K1 burden was lower ($p < 0.05$; two-tailed Mann-Whitney) at 6 h after colonization than at 24 h. No differences were found between groups at 24 h; the number of *E. coli* K1 reached a maximum (mean 8.75×10^7 CFU/g tissue) at this time point and this level of colonization persisted over the remaining period of study. No *E. coli* K1 was detected in animals before colonization or in those receiving broth. In summary, *E. coli* K1 reached climax population 24 h after administration of the bacteria in all neonates; these levels persisted in P2, P5 and P9 animals over the duration of the study (120 h) and do not lend support to a role for CR and pathogen clearance in the modulation of susceptibility to *E. coli* K1.

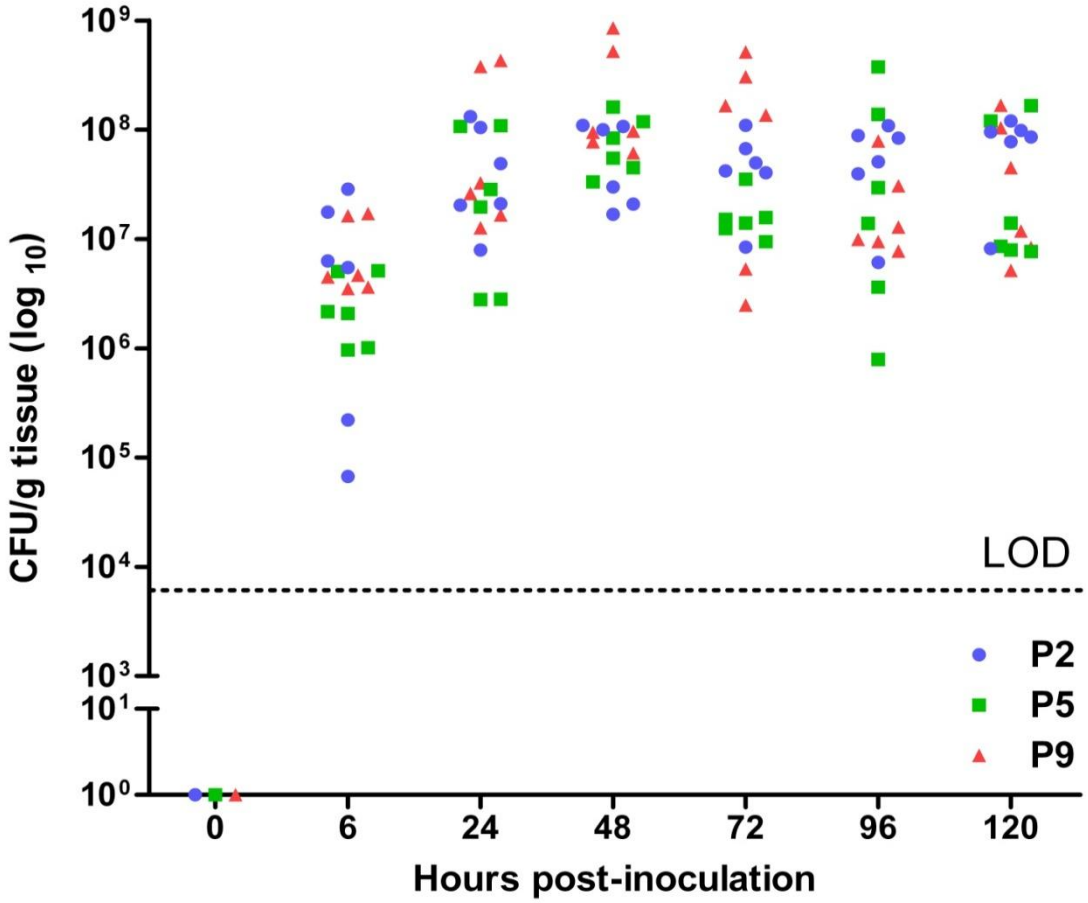


Figure 3.3: *E. coli K1 intestinal colonization. P2, P5 and P9 neonatal rats were inoculated with strain A192PP and culled at various time points after colonization. DNA was extracted from the whole intestine and E. coli K1 CFU/g tissue determined by neuS-qPCR. LOD; limit of detection.*

3.3.2 P2-P9 neonatal intestinal microbiota

The following sections describe the analysis of the neonatal intestinal microbiota at P2, P5 and P9 in order to determine if quantitative and/or qualitative differences in the composition of the microbiota influence neonatal susceptibility to *E. coli* K1 infection.

3.3.2.1 Quantitative analysis of the microbiota

The total bacterial load in the intestines of P2, P5 and P9 neonatal rats and adult rats was determined by qPCR of SSU rDNA. Four pregnant and four non-pregnant adult rats were individually caged and DNA extracted from two stool samples from each animal. Three neonates were culled *post-partum* from each litter at P2, P5 and P9 (n=12 per age-group), whole intestines (duodenum to rectum) excised and DNA extracted. qPCR was used to determine SSU rDNA copy number and results normalized to tissue or stool mass (Figure 3.4).

There were no significant differences in bacterial load between the age groups examined (p >0.52; Kruskal-Wallis). Similarly, no significant differences were found between the bacterial load of stools collected from pregnant and non-pregnant adults (p >0.38; two-tailed Mann Whitney). Comparison of the overall bacterial load of combined neonatal samples and combined adult samples indicated that there was a significant difference between these groups (p <0.01; two-tailed Kruskal-Wallis) with an average of 8.8-fold more SSU rDNA copies detected per gram of adult stool than per gram of neonatal intestinal tissue. Although this indicated that the bacterial load was higher in the adult compared to the neonatal intestine, the nature of the samples was different and this makes such comparisons difficult. Most importantly, no significant quantitative differences in the microbiota were detected over the P2-P9 period.

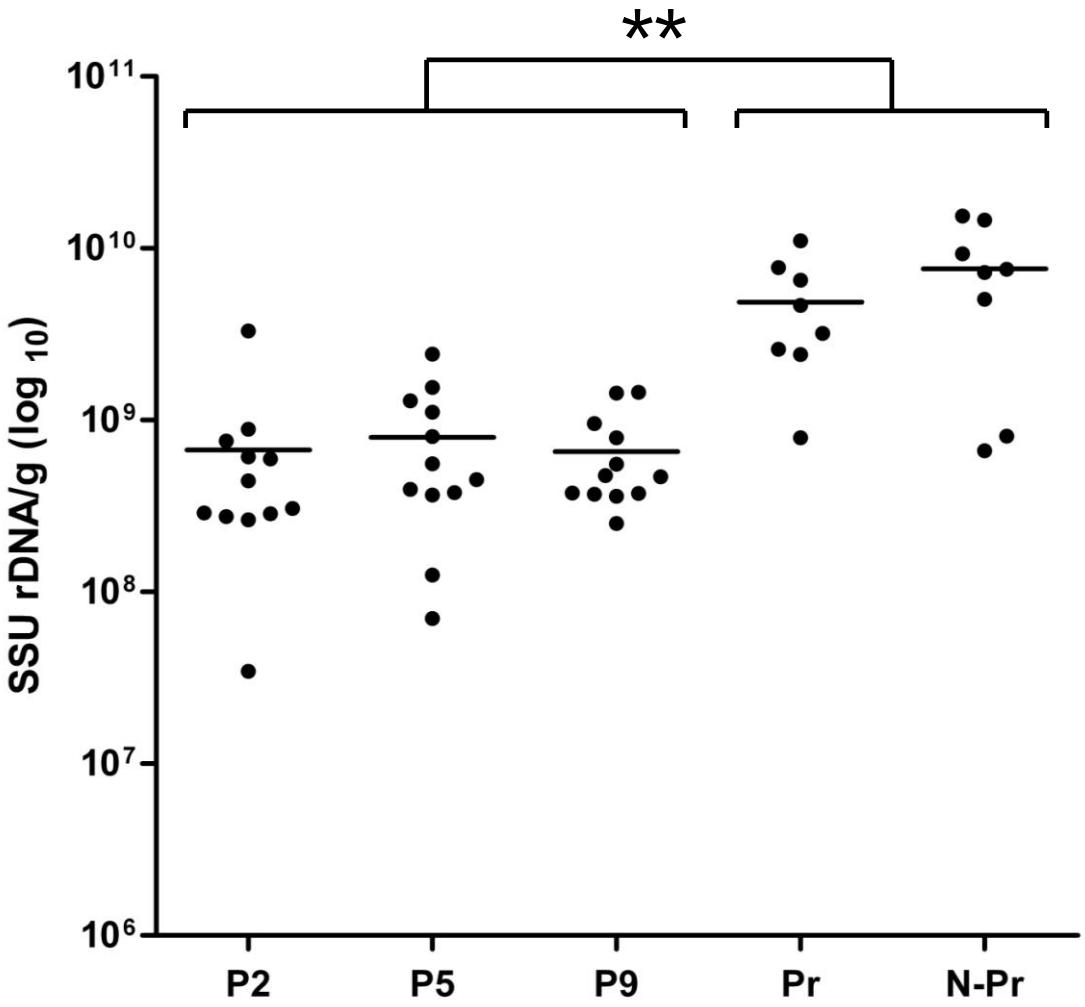


Figure 3.4: *Bacterial load in neonatal P2, P5 and P9 intestinal tissues and pregnant (Pr) and non-pregnant (N-Pr) adult stool samples. DNA was extracted from neonatal tissues, adult stool and SSU rDNA quantified by qPCR and copy number normalized to tissue/stool mass. Horizontal bars represent the mean of each group. Kruskal-Wallis test * $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

3.3.2.2 Qualitative analysis of the microbiota

The composition of the intestinal microbiota was determined in P2, P5 and P9 neonates using the SSU rDNA microarray of Palmer *et al.* (2008). The array incorporated probes with homologies to over 16000 OTU sequences in the prokMSA (http://greengenes.lbl.gov) SSU rDNA database. An OTU is defined as a group of sequences with >95% homology (DeSantis *et al.*, 2003). The array also incorporates probes for the detection of sequences conserved by related OTUs at different taxonomic levels between phylum and genus, based on the SSU rDNA region amplified by the 8FM and 1391R universal primers. Probes were labelled numerically according to their prokMSA taxonomy in phylum-species order in 1.2.3.4.5.6.7 format (Table 3.2).

prokMSA Taxonomic Level	Taxonomic Designation
1	Superkingdom
2	Phylum
3	Class
4	Order
5	Family
6	Genus
7	Species

Table 3.3: *prokMSA database taxonomic levels and equivalent traditional taxonomic designations.*

For example, *E. coli* is designated 2.28.3.27.2.007 by virtue of its species-specific probe, that is, it belongs to the Bacterial superkingdom (2), Proteobacteria phylum (2.28), Gammaproteobacteria class (2.28.3), Enterobacteriales order (2.28.3.27) and the Escherichia genus (2.28.3.27.2). Thus, any *E. coli* SSU rDNA will have sequence homology to not only its cognate level 7 (species) probe but also to the higher level taxonomic probes designed to detect broader groups of OTUs.

The DNA extracts used for these experiments were those that were employed for quantitative analysis of the neonatal and adult intestinal microbiota. Labelled SSU

rDNA amplicons from stools of individual pregnant adult rats were pooled and each pool used to hybridise a single array. In similar fashion, labelled SSU rDNA amplicons from intestinal tissues of three neonates of the same age and litter were pooled and used for hybridisation to single arrays (four single arrays for adult, P2, P5 and P9 groups). Data from neonatal array probes was filtered to remove low signals and normalized to adult array probe data to produce relative (to adult) abundance estimates for individual SSU rDNA probes.

3.3.2.2.1 Relative intestinal population overview

Processing of the neonatal array data by removal of low probe signals identified signals against 137 species level and 122 levels 2-6 taxonomic level probes and these were analysed further. The mean relative abundance of different bacterial species defined by SSU rDNA sequences from the P2, P5 and P9 neonatal microbiota and a comparison of this data with adult samples for taxonomic level 2-6 are shown in Figure 3.5. The filtered probe-set was dominated by reporters with specificity to three primary bacterial lineages, namely the Bacteriodetes, Proteobacteria and Gram-positive bacteria. In addition, the neonatal and adult microbiotas were significantly different.

The relative abundance of SSU rDNA amplicons belonging to these three phyla followed a consistent pattern in all neonatal groups. The Bacteriodetes, represented for the most part by the Bacteroides & Cytophaga level 3 taxonomic probes, were much less prevalent in neonates compared to adults. Analysis at the species level showed that several *Bacteroides* spp. that were prevalent in the adult stool microbiota were present in substantially reduced numbers in P2, P5 and P9 neonates. These species included *Bacteroides merdae*, *Bacteroides acidofaciens*, *Bacteroides fragilis*, *Bacteroides caccae*, *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron*. At the phylum level, the Proteobacteria were significantly enriched in neonatal compared to adult samples. Some differences in the sub-phylum composition of Proteobacteria were evident; neonatal samples were comparatively enriched for Alpha-, Beta- and Gammaproteobacteria and Delta- and Epsilonproteobacteria were present in reduced numbers in neonatal compared to adult samples. Analysis at the species level indicated

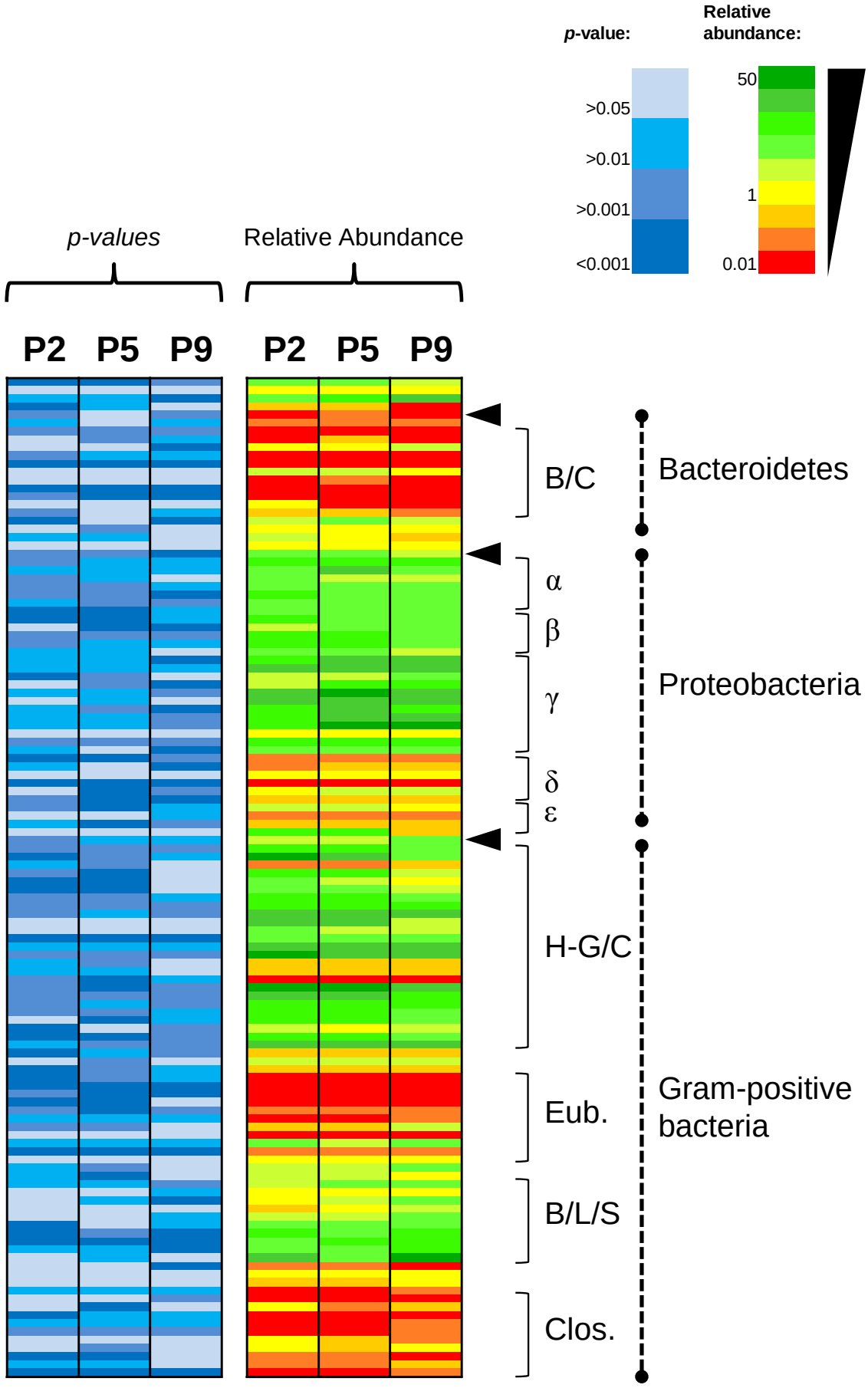

p-value:
>0.05
>0.01
>0.001
<0.001
Relative abundance:
50
1
0.01
p-values
Relative Abundance
P2
P5
P9
P2
P5
P9
B/C
α
β
γ
δ
ε
H-G/C
Eub.
B/L/S
Clos.
Bacteroidetes
Proteobacteria
Gram-positive bacteria

Figure 3.5: *Mean relative abundance of bacterial taxa detected in P2, P5 and P9 neonatal intestines. Cy5/Cy3-labelled SSU rDNA amplicons were analysed by SSU rDNA-microarrays. Filtered and normalized pooled neonatal array data was normalized to pooled adult array data to give relative (to adult) abundance of SSU rDNA species in neonatal samples (right panel). Differences between neonatal and adult probe data were determined by two-tailed Student's t-test (left panel). Columns represent data from P2, P5 and P9 neonates and rows (n=122) represent individual taxonomic probes, ranging from level 2 (phylum) to level 6 (genus) taxa. Taxonomic probes belonging to the Bacteroidetes, Proteobacteria and Gram-positive bacteria phyla (----) and the location of the level 2 probe (◄) for each of these groups are indicated. Taxonomic probes belonging to the level 3 (class) taxonomic groups Bacteroides & Cytophaga (B/C), Alpha-Epsilon (α-ε) Proteobacteria, High G/C bacteria (H-G/C), Eubacteria (Eub.), Bacillus/Lactobacillus/Streptococcus (B/L/S) and Clostridia (Clos.) are also shown (brackets).*

that the relative enrichment of the Proteobacteria was due in the main to the Gammaproteobacteria *Pasteurella* and *Pseudomonas* spp. and *E. coli*, with lower numbers of the Delta- and Epsilonproteobacteria *Desulfovibrio* and *Helicobacter* spp. Similarly, although the overall presence of Gram-positive bacteria was enriched inneonatal samples, there was substantial variation in the relative presence of different sub-phylum taxonomic groups. High G/C Gram-positive bacteria were generally enriched in the neonatal samples, with *Corynebacterium*, *Rhodococcus*, *Arthrobacter* and *Bifidobacterium* spp. Prominent. Bacteria of the Bacillus/Lactobacillus/ Streptococcus level 3 taxonomic group were also present in increased numbers in the neonate compared to the adult, with *Leuconostoc fallax*, *Lactobacillus* spp. and *Streptococcus gallolyticus* contributing to the substantial presence of this group. Conversely, the Eubacteria and Clostridial taxa, represented by species such as *Butyrivibrio fibrisolvens* and *Clostridium bifermentans*, were present in only low numbers in the neonatal microbiota . Although over 92% of the 259 reporters analysed were probes belonging to these three primary lineages these were not the only phylum level probes which produced signals above the filter cut-off value. Probes for Fusobacteria, Nitrospina and Acidobacteria phyla accounted for the remainder of these reporters, with Fusobacteria and Nitrospina present in relatively large numbers in

neonatal samples. The relative abundance of all bacteria detected by phylum level probes is shown in Figure 3.6. No significant differences were detected between these phyla, with respect to relative abundance, in the P2, P5 and P9 datasets.

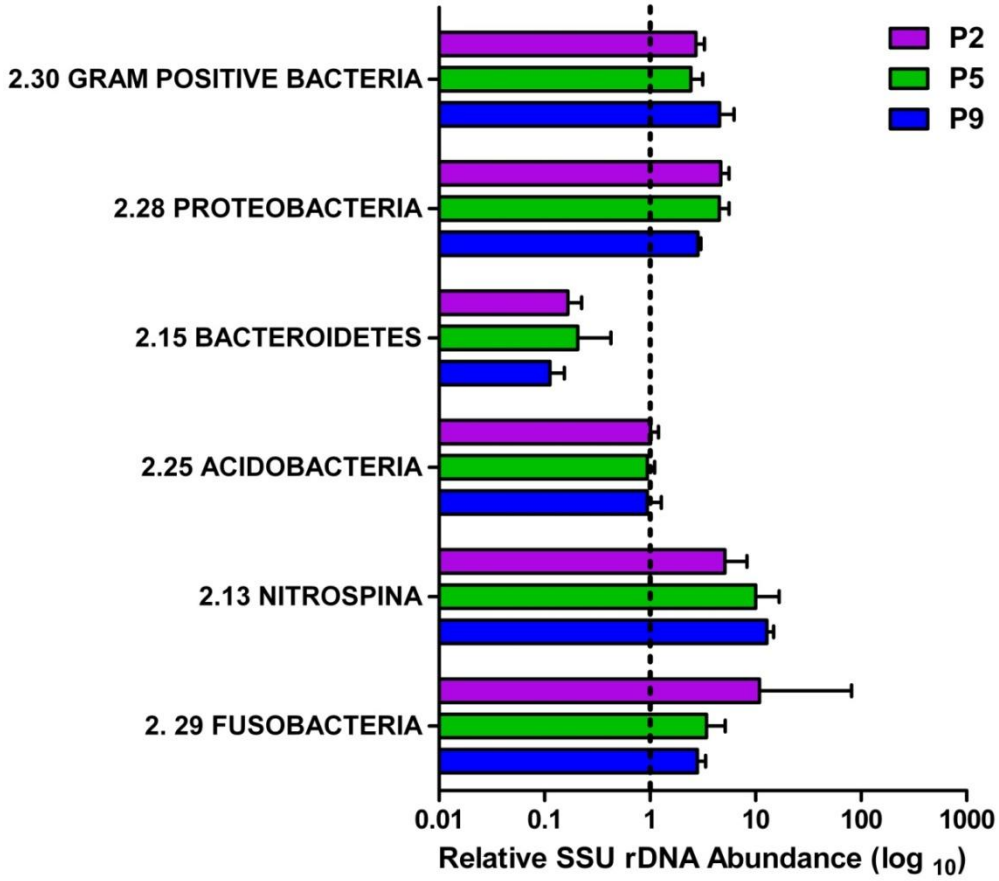


Figure 3.6: *Relative abundance of bacterial phyla detected in the P2, P5 and P9 neonatal intestinal microbiota. Data from analysis of neonatal SSU rDNA amplicons by microarray was normalized to data from adult stool samples (represented by the dashed line at x=1). Phyla are ranked according to mean Cy3/Cy5 fluorescence with the highest at the top of the figure. Error bars represent the SEM from four arrays. Numeric codes represent the prokMSA database designation for each phylum.*

Comparison of data from the three neonatal groups with adult data indicated that the neonatal intestinal microbiota was significantly different from the adult stool microbiota. Analysis of all species and higher taxonomic level probe data from P2, P5 and P9 neonatal arrays with matched probe data from adult arrays by two-tailed

Student's *t*-test showed that, of 777 comparisons, only 247 (31.79%) generated *p*-values >0.05, a clear indication that the neonatal microbiota at P2, P5 and P9 was distinct from the adult microbiota.

3.3.2.2.2 Comparison of P2, P5 and P9 intestinal microbiota

The microarray data was probed further to determine differences and similarities between the neonatal groups at sub-phylum taxonomic levels. The relationship between the neonatal datasets was assessed by Pearson correlation of mean relative SSU rDNA abundances (Figure 3.7A, B and C). The correlation between all three groups was highly significant (p <0.0001), although the strength of the correlation was variable, with the strongest between the P2 and P5 and weakest between the P2 and P9 microbiota. Analysis of individual probes by two-way ANOVA showed that two level 5 and five level 6 probes revealed significant differences in relative SSU rDNA abundance between the groups (Figure 3.7D). Over the P2-P9 period, the abundance of the *Clostridium polysaccharolyticum* and *Clostridium subterminale* subgroups increased four- and two-fold respectively. At the species level, an unclassified *Desulfovibrio* spp. increased tenfold and *Clostridium aminovalericum* and *Mycobacterium tuberculosis* increased twofold. Significant decreases of 2.5-fold were observed for *Helicobacter pylori* and *Blastochloris viridis* over the same period. A fourfold increase in *Lactobacillus casei* SSU rDNA abundance was also observed; however, this observation was not significant due to the high standard deviation at P9 (4.4-fold) for species-specific reporter probes. Overall, these results show that the P2, P5 and P9 microbiota were highly comparable, with the exception of a restricted number of species and genera which varied significantly over this period. Data generated from all probes are presented in Appendix A.

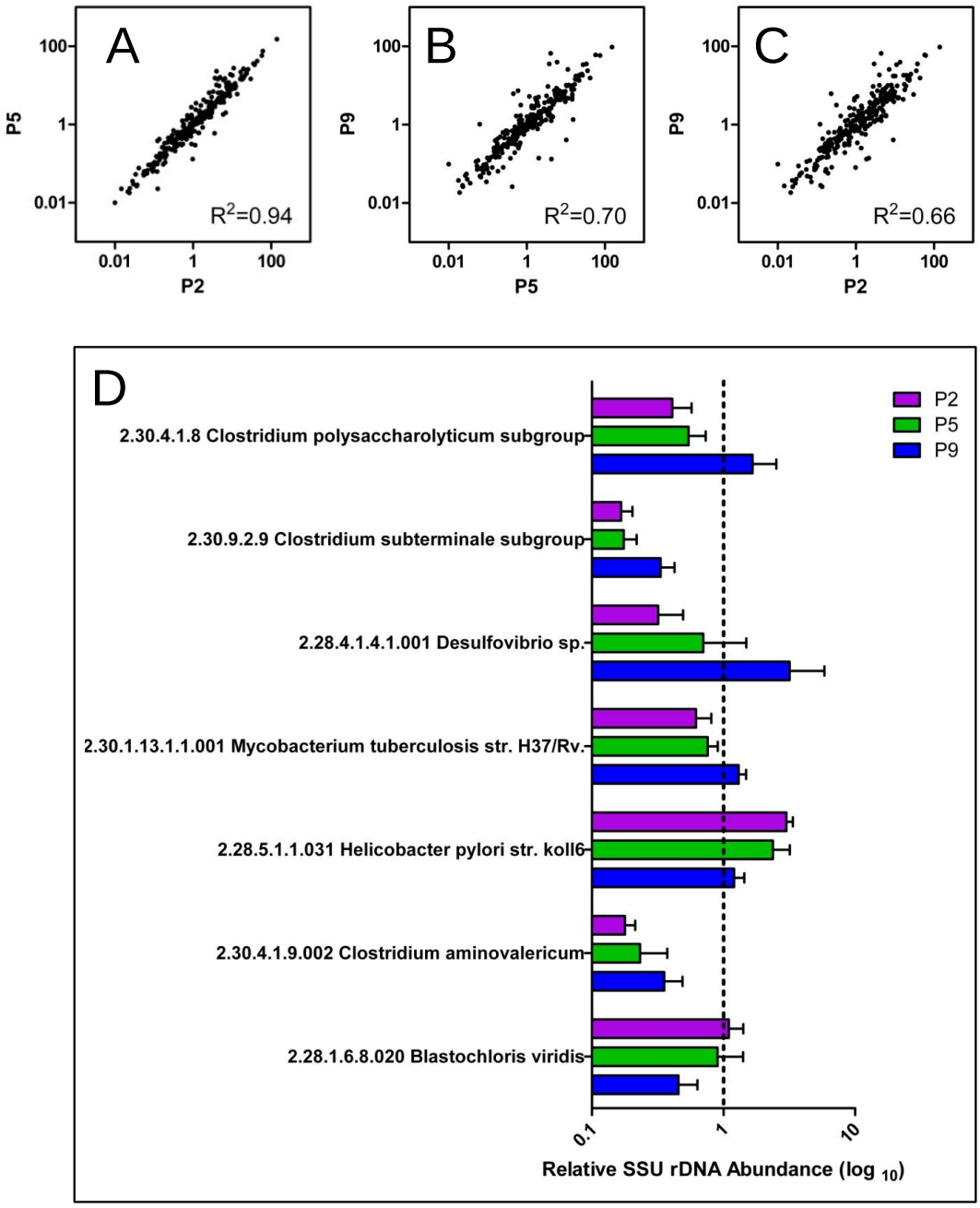


Figure 3.7: *Comparison of the P2, P5 and P9 intestinal microbiota. Mean SSU rDNA abundance detected by all probes compared between P2/P5 (A), P5/P9 (B) and P2/P9 (C) data with Pearson correlation coefficients (R^2) indicated. (D) Significant differences between P2, P5 and P9 data (two-way ANOVA). Probes ranked according to mean Cy3/Cy5 fluorescence (highest at the top of the figure). The line at x=1 represents normalized adult data. Error bars represent the SEM from four arrays. Numeric codes represent the prokMSA database designation for each taxonomic group.*

3.3.3 Antibiotic-mediated suppression of the microbiota and susceptibility to *E. coli* K1 infection

The following work assesses the impact of the microbiota on susceptibility to systemic *E. coli* K1 infection by use of antibiotic-mediated suppression of endogenous neonatal intestinal bacteria.

3.3.3.1 Antibiotic-mediated suppression of the neonatal microbiota

To optimise suppression of the intestinal microbiota, combinations of ampicillin, streptomycin, vancomycin and metronidazole were administered to groups of P7 neonatal rats. Five litters of twelve neonates each were used, with one control litter receiving water; three neonates from each of the remaining litters received either ampicillin/streptomycin (AS), ampicillin/streptomycin/vancomycin (ASV), ampicillin/ streptomycin/vancomycin/metronidazole (ASVM) or streptomycin/vancomycin/ metronidazole (SVM). All neonates were culled 24 h after antibiotic administration and DNA extracted from intestinal tissues. SSU rDNA was quantified from DNA extracts and normalized to tissue mass. The impacts of these procedures on SSU rDNA/g levels are shown in Figure 3.8; all produced a significant reduction in SSU rDNA compared to controls. However, the degree of reduction in bacterial numbers produced by each treatment was variable. Mean reductions after administration of AS, ASV, ASMV and SVM combinations were ~78%, ~86%, ~95% and ~57% respectively. Metronidazole and ampicillin contributed significantly to reductions in the bacterial population whereas vancomycin contributed little to the overall effect. The largest reduction in the size of the bacterial population was induced by ASVM treatment and this antibiotic combination was used for further study.

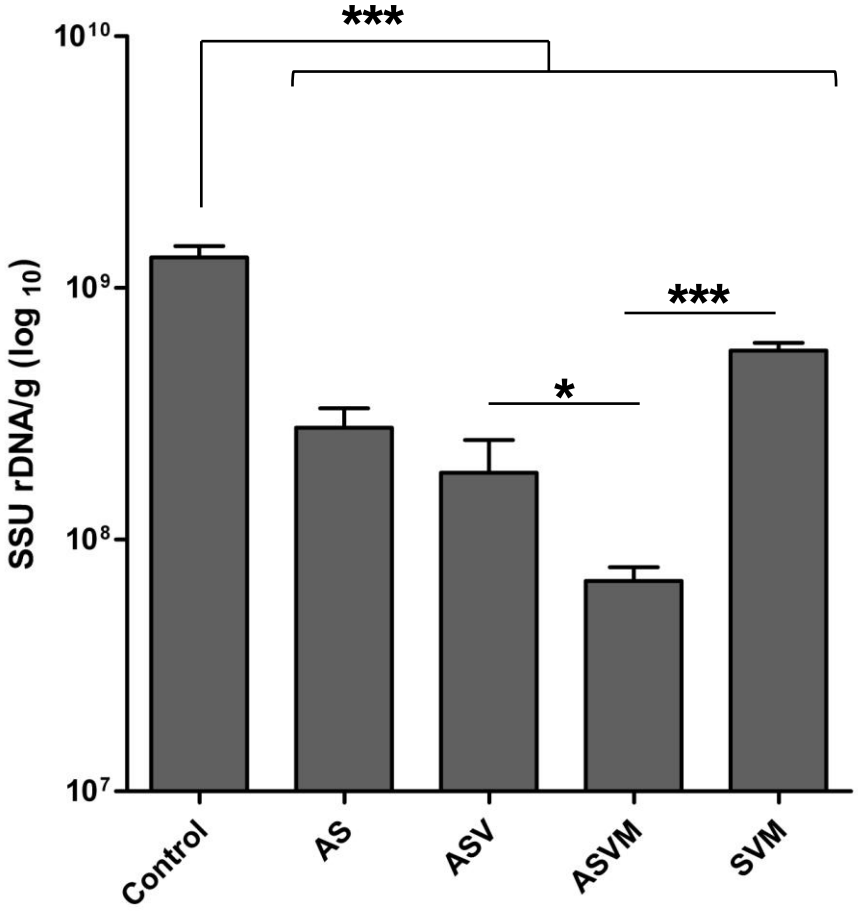


Figure 3.8: *Suppression of the microbiota by orally administered antibiotic combinations. Ampicillin (A; 10 mg), streptomycin (S; 3 mg), vancomycin (V; 10 mg) and metronidazole (M; 0.3 mg) were dissolved in water and administered to neonatal rats. Intestines were removed 24 h later and the microbiota quantified by SSU rDNA qPCR of DNA extracted from tissues. Error bars represent the SEM of twelve animals. Significant differences were determined by two-tailed Mann Whitney (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).*

3.3.3.2 Colonization of microbiota-suppressed neonates with *E. coli* K1

The MIC of A192PP was determined against ampicillin (highly susceptible), streptomycin (320 µg/mL MIC), vancomycin (1280 µg/mL) and metronidazole (1280 µg/mL). An ampicillin-resistant derivative of A192PP was constructed to enable *in vivo* use of the ASVM combination by transformation of competent A192PP with an unmodified pUC19 cloning vector encoding TEM1 β-lactamase. The transformant was

designated A192PPR and resistance to ampicillin confirmed by MIC determination (Figure 3.9). Aquisition of pUC19 increased the MIC of A192PP to ampicillin from 2.5 µg/mL to >1280 µg/mL; resistance to the other three antibiotics was not compromised comfirmed by replicate plating.

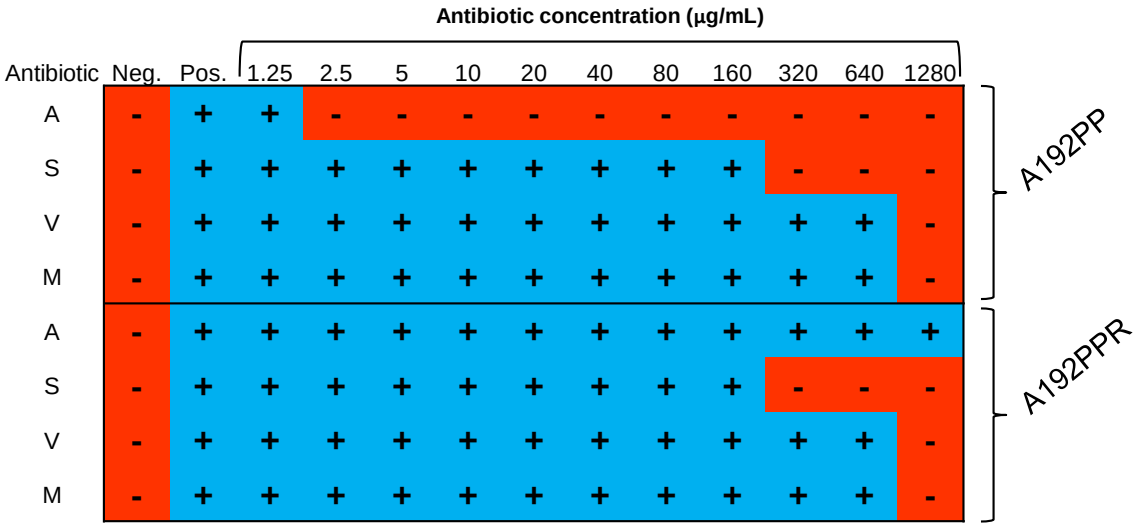


Figure 3.9: *MIC of ampicillin (A), streptomycin (S), vancomycin (V) and metronidazole (M) for strains A192PP and A192PPR. Antibiotic concentrations from 1.25-1280 µg/mL were tested along with negative (Neg.) and positive (Pos.) controls. Bacterial growth (blue; +) or lack of growth (red; -) after 24 h incubation is indicated.*

Neonatal rats undergoing ASVM treatment for suppression of the endogenous microbiota were colonized by A192PPR; four litters of twelve neonates each were used. Prior to the administration of the first dose of antibiotics four P7 neonates (1 from each litter) were culled and DNA extracted from intestinal tissues (pre-dose controls). The remaining neonates were dosed orally with each antibiotic and doses were repeated on a daily basis. Four neonates were culled on each day and DNA extracted from intestinal tissues. At P8, neonates were inoculated with 10^7 CFU of strain A192PPR. DNA extracts were analysed by SSU rDNA qPCR to determine total SSU rDNA copy number/g of tissue and by *neuS* qPCR to determine *E. coli* K1 CFU/g of tissue. *E. coli* K1 qPCR data was converted to SSU rDNA/g of tissue based on the assumption of 7

SSU rDNA copies per CFU. The mean *E. coli* K1 SSU rDNA value for each sample was subtracted from the total SSU rDNA copy number in order to determine the quantity of SSU rDNA which could not be attributed to *E. coli* K1. This remainder was assigned to the endogenous microbiota. The analysis is shown in Figure 3.10.

ASVM administration reduced the number of bacteria of the microbiota by 79.4% over the P8-P12 period; this contrasted with the 94.8% reduction determined during optimiszation of the antibiotic dosing regimen (Figure 3.9). The proportion of E. coli K1 within the bacterial population varied substantially over this period, from 25% immediately after colonization to less than 1% at P9-P10. By P13, the gut population predominated. This collapse in the endogenous bacterial population was found in all four experimental litters, occurring at P12 in one litter and P13 in the other three litters. From P13-P18, only *E. coli* K1 could be detected in the intestinal samples. These results indicated that administration of the ASVM combination of antibiotics completely suppressed the intestinal microbiota from P12/13 onwards.

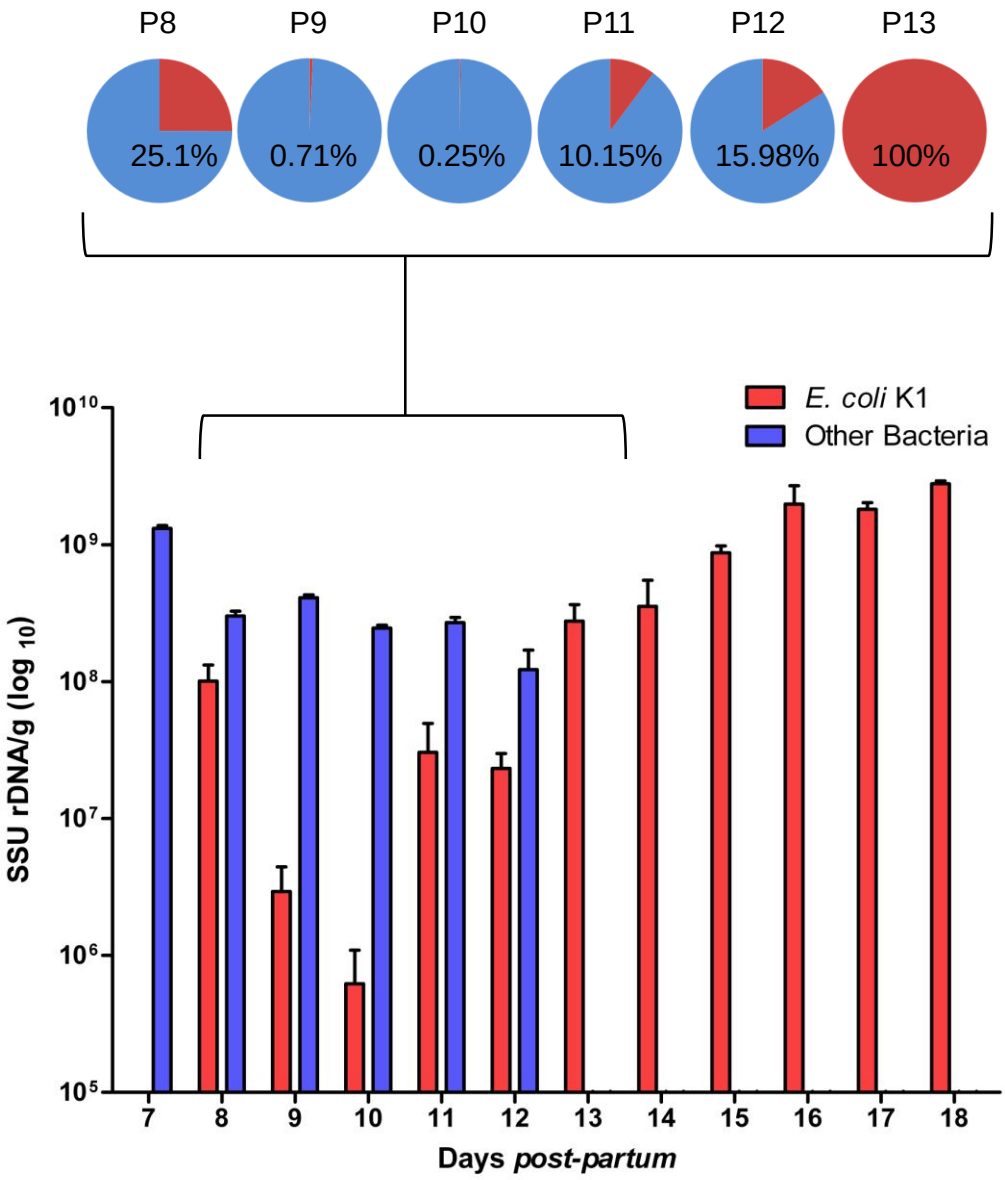


Figure 3.10: *Colonization of microbiota-suppressed neonates with E. coli K1. Neonates given daily AVSM antibiotic treatment from P7 onwards were inoculated with strain A192PPR. DNA was extracted from intestinal tissues at P7 (pre-dose control) and after colonization as indicated. Total SSU rDNA/g and E. coli K1 CFU/g were determined by qPCR. E. coli K1 SSU rDNA (7 copies/CFU) was used to determine remainder (Other Bacteria). Pie charts represent the proportion of total SSU rDNA belonging to each group from P8-P13 and %'s represent the E. coli K1 fraction. Error bars represent the SEM of four neonates.*

3.3.3.3 Impact on susceptibility to *E. coli* K1

The survival of microbiota-suppressed neonates colonized at P2 and P8 was determined. The virulence of A192PPR and the impact of ASVM treatment on survival were also assessed in susceptible neonates. Two litters of twelve neonates were used for each of these experiments; animals were colonized at P2 or P8 and survival monitored for two weeks after colonization (Figure 3.11).

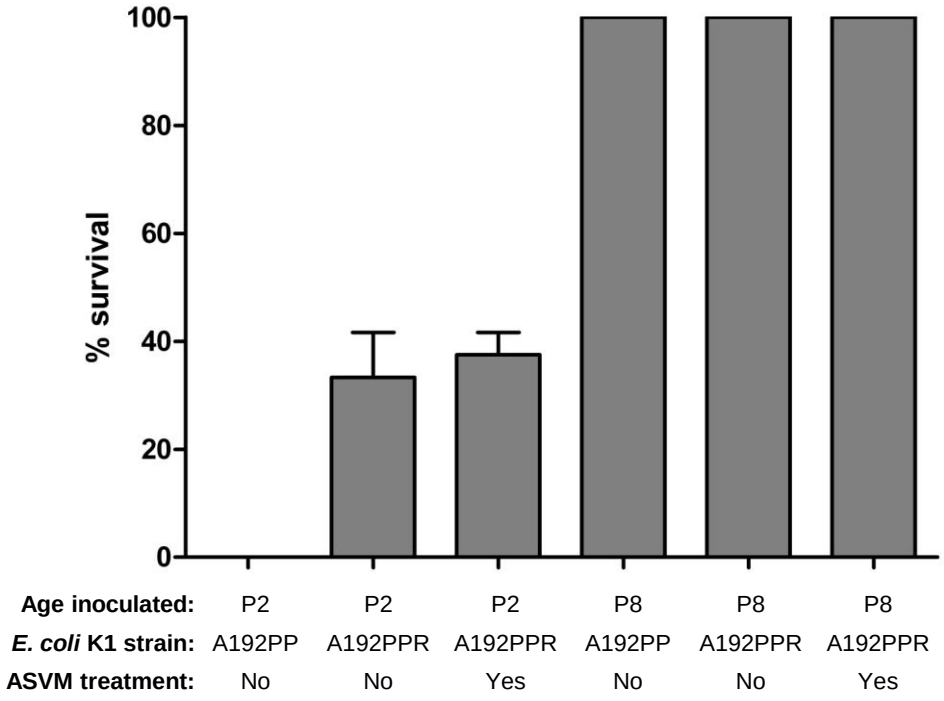


Figure 3.11: *Impact of suppression of the microbiota by antibiotic combination on survival of normally refractive neonates. Neonates given ASVM antibiotic treatment and untreated control neonates were colonized with 10^7CFU of strain A192PP at P2 or P8; survival was monitored for two weeks following colonization. Two litters of twelve were used for each condition; error bars represent the SEM. Data for untreated neonates colonized at P2 and P8 with strain A192PP are also shown.*

A192PPR was less virulent than the parent strain A192PP. However, colonization with A192PPR resulted in a substantial degree of mortality, with a mean of >66% in P2 colonized animals. ASVM administration did not influence survival, indicating that death was due to infection with A192PPR and not to antibiotic treatment.

Neonates colonized with A192PPR at P8 were refractive to systemic infection and suffered no mortality during the monitoring period. Survival of all littered neonates rate was evident in ASVM-treated and untreated experimental groups, indicating that suppression of the microbiota had no effect on susceptibility to *E. coli* K1 infection.

3.4 Discussion:

There is a sizable body of evidence that the microbiota plays an important role in the inhibition of opportunistic pathogens in the intestines. Inhibition is due to mCR mechanisms and stimulation of certain hCR mechanisms. In this chapter, I examined the relationship between the microbial population of the neonatal rat GI tract and the susceptibility of the neonatal rat to *E. coli* K1 infection. Initially, temporal aspects of *E. coli* K1 intestinal colonization in neonates differing in their age susceptibility to infection but not to GI colonization were investigated.

E. coli K1 may colonize the GI tract of hosts that are naturally refractive to systemic *E. coli* K1 infection, illustrated by the high rate of commensular *E. coli* K1 carriage in the human adult population (Sarff *et al.*, 1975) and from studies in animals (Glode *et al.*, 1977; Bortolussi *et al.*, 1978; Pluschke & Pelkonen, 1988). As reported in Chapter 2, *E. coli* K1 intestinal colonization can be induced in P9 neonatal and adult rats, although quantitative aspects of intestinal colonization of susceptible and refractive neonates were not assessed and no conclusions could therefore be drawn regarding differences in colonization rates between these two groups . No differences in the *E. coli* K1 burden after colonization at P2, P5 or P9 could be demonstrated at any time point and the temporal development of the K1 population was very similar in these age groups. This suggests that CR mechanisms do not affect *E. coli* K1 colonization and survival within the GI tract and the pathogen is not cleared from the intestines of susceptible or refractive neonates by the endogenous microbiota. The stabilization of the *E. coli* K1 intestinal load at 24 h after colonization suggests that there is an upper limit to the size or growth of the bacterium in the neonatal intestine. The *E. coli* K1 population climaxed immediately prior to the bacterial translocation window (24-72 h after colonization) reported in the previous chapter. Translocation across the BBB requires a threshold bacterial load of around 10^3 CFU/mL in the bloodstream (Dietzman *et al.*, 1974) and it is possible that there is also a threshold of similar dimension for translocation from the gut lumen to the bloodstream.

The lack of CR or pathogen clearance does not preclude a role for the microbiota in the prevention of dissemination of the pathogen from the intestinal tissues. For

example, some microbiota-pathogen interactions can modulate the virulence of the pathogen without preventing its growth in the intestine: commensal *E. coli* strain Nissle 1917 inhibits the invasive mechanisms of EIEC strains and *Listeria monocytogenes* (Altenhoefer *et al.*, 2004). *Bifidobacterium* and *Lactobacillus* spp, are able to inhibit the function of VFs expressed by pathogenic *Salmonella* spp. (Bernet *et al.*, 1993; Coconnier *et al.*, 2000). In similar fashion, species closely related to some pathogens can determine susceptibility to that pathogen (Stecher *et al.*, 2010). Susceptible neonates may lack species such as *B. thetaiotaomicron* and segmented filamentous bacteria that induce the secretion of AMPs and maintain luminal bacteria at a distance from the intestinal epithelium (Keilbaugh *et al.*, 2005). These studies informed the quantitative and qualitative assessment of the P2, P5 and P9 intestinal microbiota that were undertaken and described in this chapter.

The intestinal microbiota of the three neonatal age groups employed in this study were quantitatively and qualitatively different to the adult microbiota. Facultative anaerobes, including Gammaproteobacteria and taxonomic groups within the Firmicutes and High G/C (Actinobacteria) phyla, were prominent members of the neonatal GI microbiota and were complemented by a concomitant paucity of strict anaerobes of the phyla Firmicutes and the Bacteroidetes. Similar profiles have been found by other investigators (Favier *et al.*, 2002; Palmer *et al.*, 2008), supporting the validity of the microarray and data analysis methods employed in the current study and indicating that the neonatal rat microbiota was broadly comparable to its human equivalent at the higher taxonomic levels. *Lactobacillus* spp, *Bifidobacterium* spp. and endogenous *E. coli* were prominent members of the neonatal microbiota. Furthermore, the neonatal microbiota was deficient in AMP-inducing *B. thetaiotaomicron* in comparison to the adult microbiota. These bacteria are unlikely to have played a role in determination of neonatal susceptibility to *E. coli* K1 over the P2-P9 period. In similar fashion, the lack of absolute quantitative differences between the neonatal cohorts indicated that the number of endogenous bacteria did not influence susceptibility to *E. coli* K1.

Some taxa (including several Clostridial groups) were found to vary, in quantitiative terms, over the P2-P9 period. The *C. polysaccharolyticum* subgroup is a poorly characterized genus comprising fifty known OTUs. None of the OTU-specific probes for this subgroup passed the probe filter, indicating that the relative increase in numbers of members of this group was due to an unidentified component(s) of this

genus. The group archetype is a butyrate producing fermenter of cellulose and starch originally isolated from ruminant animals (Van Glyswyk, 1980). Butyrate inhibits the growth of some pathogens (Hopkins & Macfarlane, 2003) and reduces *E. coli* translocation of enterocytes *in vitro* (Lewis *et al.*, 2010). Numbers of the *C. subterminale* subgroup (24 OTUs) and *C. aminovalericum* also increased over P2-P9. Both are common constituents of the mammalian intestinal microbiota (Lee *et al.*, 1991), but any specific mechanism by which they might affect susceptibility to *E. coli* K1 is not immediately apparent. However, an investigation by Itoh & Freter (1989) indicated that Clostridial species can control colonic *E. coli* populations and there is a possibility that these organisms have the potential to modulate susceptibility to *E. coli* K1.

A *Desulfovibrio* species increased in numbers substantially over the P2-P9 period. These bacteria are common constituents of the intestinal microbiota (Gibson *et al.*, 1993) and are characterized by the metabolism of sulphate to toxic hydrogen sulphide. There is, however, no evidence that they confer any protective effects on the host. Surprisingly, another species which increased significantly during the period of observation was *Mycobacterium tuberculosis*, a pathogen associated with chronic pulmonary infections but which can survive and cause disease in the intestinal tract (reviewed by Donoghue & Holton, 2009). Two species of bacteria, *H. pylori* and *B. viridis*, showed a significant decline over P2-P9. The relevance and accuracy of the *B. viridis* result is suspect as the relevant probe only fractionally escaped the filtering method and the species is not associated with enteric environments. *H. pylori* is a common constituent of the upper GI tract and can be vertically acquired by the neonate following vertical transmission from the mother (Solnick *et al.*, 2003). Again, there is no known mechanistic basis by which an alteration in this bacterial population could influence susceptibility of the neonate to *E. coli* K1 infection.

Suppression of the neonatal microbiota by daily oral administration of antibiotics produced data that was broadly comparable to results published by other investigators (Croswell *et al.*, 2009). The substantial decrease in the microboita after several days of treatment has also been previously reported (Croswell *et al.*, 2009; Rakoff-Nahoum *et al.*, 2004; Fagarasan *et al.*, 2002). The variable degree of microbiota suppression observed for the different antibiotic combinations may be attributable to the antibacterial spectra of the individual antibiotics employed. Both ampicillin and

streptomycin are broad spectrum and have a significant impact on the microbiota. The statistically insignificant impact of vancomycin may be due to the fact that its mode of action restricts its activity against Gram-negative bacteria. The antibacterial spectrum of metranidazole is limited to anaerobic bacteria; however, this would be unlikely to constrain its activity in the anaerobic environment of the GI lumen.

The resistance of A192PPR to the antibiotics used in this study may have provided the strain with an advantage over drug-susceptible members of the GI microbiota under the experimental conditions employed and this may have contributed to the degree of suppression of the microbiota. This model circumvents the potential pitfalls of a GF model, as the neonates have been exposed to the normal microbiota prior to P7 and would have received the developmental cues prompted by acquisition of commensular intestinal bacteria. The conditions used allowed analysis of the effects induced by the absence of the microbiota on susceptibility to *E. coli* K1 infection. The lack of mortality observed in naturally refractive (i.e. P8 or older) neonates is evidence that the loss of the microbiota does not affect susceptibility to *E. coli* K1. However, the decrease in A192PPR virulence represents a potential cause for concern, as it implies that the transformation process compromised the virulence of the strain. A192PPR may, therefore, be less able to survive in extra-intestinal niches of refractive neonates.

CHAPTER 4

DEVELOPMENT OF HOST INTESTINAL TISSUES & RESPONSE TO *E. COLI* K1 COLONIZATION

4.1 Introduction

The post-natal development of the neonatal intestinal tissues contributes to the dynamic nature of the enteric environment. This developmental flux represents a variable which could modulate the capacity of *E. coli* K1 to translocate from the intestinal lumen into the systemic circulation. The K1 capsule provides the pathogen with a defensive mechanism which inhibits activation of the adaptive arm of the intestinal immune system. Therefore, development of the innate intestinal defences is likely to be a factor that impacts on the determination of the susceptibility of the host to *E. coli* K1 infection.

The *post-partum* increase in Paneth cell differentiation and AMP secretion (Mallow *et al.*, 1996; Bry *et al.*, 1994) represents a key element in the development of innate intestinal defences. The classic AMP family are the α-defensins, a group of small, structurally conserved peptides with strong antibacterial activity against a broad spectrum of bacterial species. The enteric members of this family are especially potent in terms of their bactericidal activity (Ericksen *et al.*, 2005). Mature α-defensin peptides are 29-39 amino acids in length and possess several features which are vital to their function. They have an overall cationic charge, conferred by multiple arginine residues, are amphiphilic and also possess six conserved cysteine residues mediating the formation of three disulphide bridges. The polar properties of the mature peptide facilitate interaction and insertion into negatively charged bacterial membranes. The tertiary structure of these peptides mediates the formation of a pore-like structure which depolarizes the target membrane, induces the leakage of ions and ATP and inhibits bacterial respiration (reviewed by White *et al.*, 1995). Enteric α-defensins can also act as paracrine regulators of the inflammatory response by stimulation of IL-8 secretion (Lin *et al.*, 2004) and inhibition of IL-1β release from activated monocytes (Shi *et al.*, 2007).

The mucus layer is another essential aspect of innate enteric defence and functions to maintain luminal bacteria at a safe distance from the intestinal epithelia. Gel forming mucins, such as Muc2, are vital in realizing this function; however, the exact mechanism by which bacterial/epithelial separation is achieved in the different intestinal compartments is variable. In the small intestine, the mucus layer acts to

maintain Paneth cell-secreted AMPs in close proximity to the intestinal enterocytes (Vaishnava *et al.*, 2011; reviewed by Johansson & Hansson, 2011). In this manner, the small intestinal mucus layer maintains bacterial separation using a bactericidal gradient, with the highest concentration of AMP closest to the epithelium. The colon lacks Paneth cells and the colonic mucus layer thus relies on an alternate mechanism of bacterial separation. This mechanism comprises a stratified physical exclusion barrier that the majority of intestinal bacteria are unable to penetrate (Johansson *et al.*, 2008). The barrier is primarily composed of Muc2 arranged in layered sheets of hexagonal 1 μm diameter rings, with each ring composed of twelve Muc2 monomers (Ambort *et al.*, 2012). The developmental regulation of AMP secretion implies that the barrier function provided by the small intestinal mucus layer is developmentally regulated *post-partum*. Conversely, nothing is known of the developmental state of the colonic mucus layer at birth and therefore it too may be developmentally immature in the immediate post-natal period. Muc2 is expressed in the foetal colon (Chambers *et al.*, 1994); however, there are other factors which may be vital to the formation of the stratified exclusion barrier. These include two other major goblet cell-secreted proteins; trefoil factor 3 (Tff3) and Fc-gamma binding protein (Fcgbp).

The trefoil family peptides (Tff1-3) are so named due to their characteristic trefoil domain. This consists of a triple loop 'clover-leaf' structure which is maintained by three cysteine-cysteine disulphide bonds. *Tff2* has two trefoil domains whereas Tff1 and Tff3 have one each (Thim, 1989; 1997). Trefoil peptides are the second most abundant protein found in mucin-secreting cells and several studies have indicated that they play key protective roles in the GI tract. These include a motogenic function, which is required to stimulate epithelial healing after damage, and regulation of the inflammatory response (Playford *et al.*, 1995; Tran *et al.*, 1999; Kurt-Jones *et al.*, 2007). Furthermore, all trefoil peptides bind to Paneth cells and *Tff2*-KO mice differentially express enteric α-defensins and proteins involved in the presentation of antigens to immune cells (Poulsen *et al.*, 2003; Baus-Loncar *et al.*, 2005). Trefoil peptides alter the viscoelastic properties of the mucus layer by complexing with mucins (Figure 4.1) and have a synergistic protective effect on the intestinal epithelium (Thim *et al.*, 2004; Kjellev *et al.*, 2006; Playford *et al.*, 2006). Trefoil peptides interact non-covalently with mucins through the cysteine-rich von Willebrand Factor C (vWFC) domain of the mucin molecule (Tomasetto *et al.*, 2000) and also form disulphide bonds with Fcgbp

(Albert *et al.*, 2010). Fcgbp contains multiple vWFD domains and covalently binds to Muc2 (Johansson *et al.*, 2011). The Muc2/Tff3/Fcgbp complex can be purified by co-immunoprecipitation and visualized in the stratified colonic mucous layer (Yu *et al.*, 2011). The interactions of mucin, trefoil factors and Fcgbp are thus likely to contribute to the stratified colonic mucous barrier. Tff3 is expressed relatively late in gestation and expression increases substantially *post-partum*. This may indicate that the early neonatal barrier may not be as robust as that of the older neonate or adult (Lin *et al.*, 1999, Mashimo *et al.*, 1995).

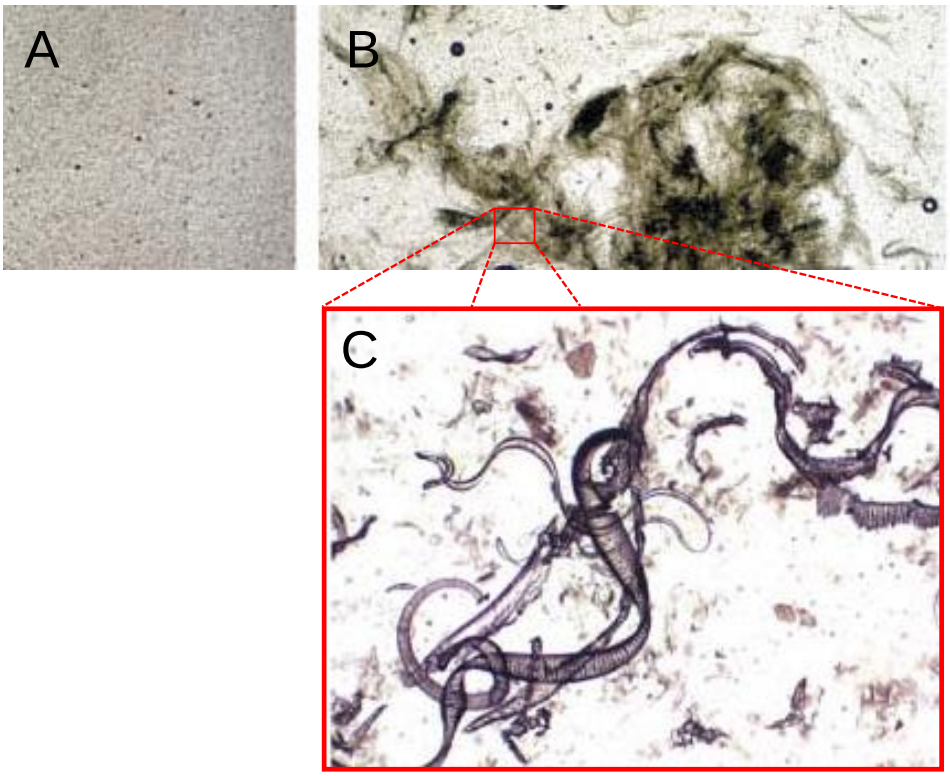


Figure 4.1: *Trefoil factor 2 complexed with mucins. Images are of mucin (A), trefoil factor 2 plus mucin (B) and a magnified image of the long chain Tff2/mucin complex (C). Images adapted from Thim et al. (2004).*

The neonate is distinctly susceptible to a range of inflammatory conditions such as pneumonia, meningitis and NEC. The implication of this susceptibility is that regulation of the neonatal inflammatory response and/or the effector leukocytes summoned to the site of inflammation are immature. This would result in either an overly prolonged or incapacitated response to inflammatory stimuli. The neonatal innate immune response is demonstrably distinct from that of the adult (reviewed by Levy, 2007); however, whether the neonate is hyper- or hypo-responsive to inflammatory

stimuli remains a source of controversy. Much of the research in this arena has focused on the *ex-vivo* secretion of pro-inflammatory cytokines by circulatory leukocytes. Some investigators have reported a significant deficiency in IL-1β, IL-6 and TNF-α secretion, as well as the reduced presence of LPS responsive CD14 and TLR4 receptors in neonatal compared to adult leukocytes (Peters *et al.*, 1993; Levy *et al.*, 2006; Qing *et al.*, 1995; Förster-Waldl *et al.*, 2005). However, other investigators have reported enhanced production of these cytokines and receptors under similar experimental conditions (Berner *et al.*, 2002; Yerkovich *et al.*, 2007; Tatad *et al.*, 2007).

There is *ex-vivo* evidence that the pre-term and very young neonatal intestine tissue is hyper-responsive to inflammatory stimuli (Nanthakumar *et al.*, 2000; Lotz *et al.*, 2006; Okogbule-Wonodi *et al.*, 2012). *In-vivo* data tends to support the hyper-responsive neonatal phenotype (Cusumano *et al.*, 1997; Zhao *et al.*, 2008). Furthermore, the neutrophil population of the neonate is qualitatively distinct from that of the adult and shows reduced production of key molecular armaments against microbial pathogens. These include the capacity to produce reactive oxygen species, lactoferrin, lysozyme and BPI (Ambruso *et al.*, 1984; Levy *et al.*, 1999). It is possible that the hyper-inflammatory response of the neonate may be required in order to overcome these neutrophil deficiencies. However, this carries the potential cost that inflammation may damage host tissues.

The transcriptome is defined as the total mRNA produced by individual cells or whole multicellular tissues and varies substantially in response to differential stimuli. As the functional template for protein synthesis, the transcriptome can be taken as an indirect measure of the proteome. This assumption has been validated *in-vivo* but has several caveats, including the inability to detect post-translational regulation or to distinguish mRNA associated with active polysomal from inactive monosomal ribosomes (Scherl *et al.*, 2005; Kislinger *et al.*, 2006; reviewed by Hegde *et al.*, 2003). Comparative analysis of the transcriptome provides a powerful tool for the assessment of the reactions of cells or tissues to stimuli, such as microbial infection. This approach can also be used to evaluate developmental gene regulation. This chapter describes experiments designed to assess the development of the neonatal intestinal tissues over the period that resistance to systemic *E. coli* K1 infection increases. In addition, the response of intestinal tissues to colonization by the pathogen was also characterized.

4.2 Materials & Methods

Unless otherwise indicated, in the following sections all growth media were purchased from Oxoid Ltd, all chemicals, reagents and enzymes were from Sigma-Aldrich and all oligonucleotides were synthesised by and purchased from Eurofins MWG Operon. This section describes methods which are specific to the results described in this chapter; some methods used in Chapter 2 were also employed.

4.2.1 Oligonucleotides

Multiple oligonucleotides were used in experiments described here. Probe and competitor sequences used in NFκB electrophoretic mobility shift assays (EMSA) are detailed in Table 4.1. Primer pairs used to amplify fragments of genes analysed by RT-PCR are detailed in Table 4.2.

Name	Strand	Sequence (5'-3')
NFκB wt Cy5	sense	**CY5**-AGTTGAGGGGACTTTCCCAGGC
	antisense	**CY5**-GCCTGGGAAAGTCCCCTCAACT
NFκB wt	sense	AGTTGAGGGGACTTTCCCAGGC
	antisense	GCCTGGGAAAGTCCCCTCAACT
NFκB mut	sense	AGTTGAGGCGACTTTCCCAGGC
	antisense	GCCTGGGAAAGTCCGCTCAACT

Table 4.1: *Sense and antisense strand sequences of the NFκB wild-type (wt) Cy5-conjugated probe with wild-type and mutant (mut) competitors. The NFκB binding site is underlined on the wild-type sequences and the mutated base pair is underlined on the mutant sequences.*

Target	Primer	Sequence (5'-3')
Rps23	F	TGTGTCAGGGTGCAGCTCATTAAGAACG
	R	CTTTGCGACCAAATCCAGCAACCAGAAC
Defa-rs1	F	GACCAGGATGTGTCTGTCTCCTTTG
	R	TGTGGACCTTGATAGCCGAATGC
Pdcd4	F	AGAAGTGGAGTAGCTGTGCCCACCAGTC
	R	CCCTTGCCTCCTGCACCACCTTTCTTTG
Clic4	F	AAAGGCATGACGGGCATCTGGAGATACC
	R	GTCACTGTACGCGATTTCCACCTCCTTG
Cav	F	GCAAGTGTACGACGCGCACACCAAGGAG
	R	CCAGATGAGCGCCATAGGGATGCCGAAG
Afp	F	GTGAGGGACTGGCCGACATTTACATTGG
	R	GTGATGCAGAGCCTCCTGTTGGAATACG
Amy2	F	CAGAAATTGTCGTCTGTCTGGCCTTCTG
	R	CAAGTCTGAACCCTGCTACACCAATGTC
RT1-Aw2	F	GGTCAGGGTGATGTCAGCAGGGTAGAAG
	R	GCTCAGCAGATACCTGGAGCAAGGGAAG
Btg2	F	ACTGCTCCTGCCCAGCATCATCTGGTTC
	R	ATCCAAGGGCTCCGGCTATCGCTGTATC
Cald1	F	CTTGCTTCTGCCGCAGCCTTTCCTGTCG
	R	CCAGGCGCATCTTGCTCAGCGCATTTCG
Tff2	F	GGCATCACCAGTGACCAGTGCTTTAATC
	R	GCAGTGCCCTTCAGTAGTGACAATCATC
Ins2	F	AAGTGACCAGCTACAGTCGGAAACCATC
	R	AGCTTCCACCAAGTGAGAACCACAAAGG
Defa24	F	TGATGAGCAGCCAGGGAAAGAG
	R	TCAGCGGCAACAGAGTATGAGC
NFκB	F	CAAGAACAGCAAGGCAGCACTCC
	R	TGTAGAGGTGTCGTCCCATCGTAGG
Cebp/β	F	GCCGCCTTTAGACCCATGGAAGTG
	R	AACCGTAGTCGGACGGCTTCTTGC
Muc2	F	CCTCAACGGCATCCATTCC
	R	AGGTGGGTAGCGAGTATCC

Table 4.2: *Primers used to amplify gene fragments in RT-PCR. Target genes and forward (F) and reverse (R) primer sequences are detailed.*

RT-PCR primer pairs were designed using Clone Manager Suite (v.9) software (Scientific & Educational Software). *Rattus norvegicus* gene-specific mRNA sequences were obtained from the NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) and used as design templates. Primer pairs spanning known exon-exon boundaries were preferentially selected. Primer specificity was examined by testing primer pairs using Primer-BLAST (www.ncbi.nlm.nih.gov /tools/primer-blast) with primer pair specificity checking parameters set to all deposited bacterial and *Rattus norvegicus* sequences in all DNA sequence repository databases.

4.2.2 RNA extraction

Tissue RNA was stabilized by submerging excised tissues in five volumes of RNAlater tissue storage reagent (Ambion) and stored at 4 °C for at least 24 h prior to nucleic acid extraction. Total RNA was extracted from RNA-stabilized intestinal tissues using RNeasy Midi-Kits (Qiagen) according to the manufacturer's instructions. The compositions of buffers utilized in the extraction process were proprietary information unless otherwise detailed. The flow-through produced by centrifugation steps was discarded unless otherwise indicated. Tissues were transferred from RNAlater solution to 7.5 mL of lysis Buffer RLT containing 1% (v/v) β-mercaptoethanol. Tissues were disrupted and homogenized in lysis buffer using an Ultra-Turrax T-10 rotor-stator homogenizer. The homogenizer blade was washed once in 70% (v/v) ethanol and three times in sterile PBS between samples. Homogenates were centrifuged at 5000 x *g* for 20 min. Centrifugation resulted in a pellet and a fatty upper layer, both of which were selectively removed using a pipette. The supernatant was mixed with 7.5 mL of 70% (v/v) ethanol, applied to spin-columns and centrifuged at 5000 x *g* for 10 min. Columns were washed with Buffer RW1 and centrifuged at 5000 x *g* for 5 min. Contaminating DNA was degraded by on-column DNase digestion using RNase-free DNase Set kits (Qiagen) according to the manufacturer's instructions. DNase I (375 U/mL) was applied to each column and incubated at room temperature for 15 min. Columns were washed once with Buffer RW1 and twice with Buffer RPE. RNA was eluted from the columns in 150 µL of RNase-free H_2O, transferred to RNase-free microcentrifuge tubes (Ambion) and stored at -80 °C. RNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Scientific). RNA (1 µg) was mixed with an

appropriate volume of 6 x Gel Loading Buffer (0.05% [w/v] bromophenol blue, 40% [w/v] sucrose, 0.1 M pH 8 EDTA, 0.5% [w/v] SDS) and resolved by agarose gel electrophoresis as described in section 2.2.10. Gels were visualized using a Molecular Imager FX system (Bio-Rad) set to detect UV fluorescence. Images were used to assess RNA integrity and the presence of contaminating genomic DNA (Figure 4.2).

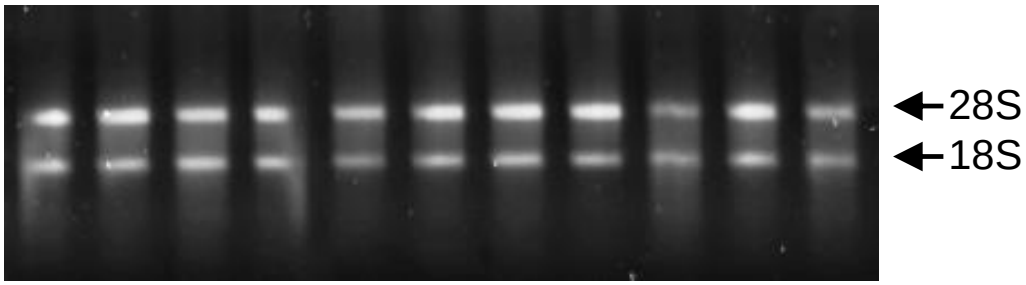


Figure 4.2: *Assessment of RNA integrity and genomic DNA contamination by agarose gel electrophoresis. 1 µg of RNA extractions was loaded onto 1% (w/v) agarose gels and resolved by electrophoresis. Intact 28S and 18S rRNA bands (indicated) were used to assess integrity. No large genomic DNA fragments were detected in these samples.*

4.2.3 Protein extraction

Protein was extracted from intestinal tissue samples under denaturing conditions. Freshly excised tissues were transferred to 4 mL of ice-cold protein extraction buffer (1% [v/v] NP-40, 1% [v/v] Tween-20, 10 mM pH 7.4 Tris-HCl, 1mM EDTA in PBS) supplemented with 1 x Complete Mini Protease Inhibitor Cocktail (Roche). Samples were weighed and homogenized on ice using an Ultra-Turrax T-10 homogenizer (IKA-Werke). The homogenizer blade was washed once in 70% (v/v) ethanol and three times in sterile PBS between samples. Tissue homogenate (0.96 mL) was mixed with 3 mL of 10 M urea (7.5 M final concentration) and 40 µL of 1 M dithiothreitol (DTT; final concentration 100 mM). Samples were incubated for 24 h on a slowly rotating orbital shaker at 4 °C. Denatured tissue homogenates were centrifuged at 1500 x *g* for 10 min at 4 °C and the supernatant aspirated and retained. The protein content of the extract was measured according to Bradford (1976). Concentrated Protein Assay Dye (Bio-Rad) was diluted 1:5 with H_2O to make a working solution of Bradford reagent. A series

of twofold dilutions of bovine serum albumin (BSA; 1 mg/mL) was prepared over the range 62.5-1000 µg/mL. Aliquots (20 µL) of these standard solutions or protein extracts were added to 1 mL of Bradford working solution and thoroughly mixed. The OD_{595} of the mixture was measured with a Lambda 25 spectrophotometer (Perkin-Elmer). Standard OD_{595} values were used to construct a standard curve and in order to determine sample protein concentration.

4.2.4 Preparation of single cell suspensions from tissue

Freshly excised intestinal tissues were washed in 2 mL of ice-cold PBS. Washed tissues were transferred to 4.7 mL of HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ in ddH_2O) supplemented with DNase I (80 U/mL) and collagenase (2 mg/mL). Tissues were briefly (~10 s) homogenized on ice using an Ultra-Turrax T-10 homogenizer (IKA-Werke). The homogenizer blade was washed once in 70% (v/v) ethanol and three times in sterile PBS between samples. Homogenates were incubated at 37 °C in an orbiting incubator rotating at 100 rpm for 30 min followed by another brief homogenization. BD Falcon Cell Strainers (100 µm; BD Biosciences) were placed in 50 mL collection tubes and the tissue homogenate applied directly to the cell strainer filter. Filters were washed with 3 mL of HEPES buffer. Single cell suspensions (the filtrate) were centrifuged at 500 x *g* for 10 min at 4 °C to pellet cells. All samples were kept on ice at all times unless otherwise specified. All buffers were filter-sterilized using 0.22 µm MILLEX GP filters (Millipore).

4.2.5 Nuclear protein extraction

Tissue cell pellets were suspended in 5 mL nuclear extraction buffer (0.32 M Sucrose, 10 mM pH 7.4 Tris-HCl, 3mM $CaCl_2$, 2 mM MgOAc, 0.1 mM EDTA, 1 mM DTT) supplemented with 0.5% (v/v) NP-40 and 1 x Complete Mini Protease Inhibitor Cocktail (Roche) and mixed well to allow lysis of cell membranes. The suspension was centrifuged at 500 x *g* for 5 min at 4 °C to pellet cell nuclei. The cytoplasmic fraction (supernatant) was aspirated and stored at -80 °C. Nuclei pellets were washed three times in nuclear extraction buffer. Protein was extracted from washed cell nuclei by

suspending pellets in 1.5 mL hypotonic buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25% [v/v] glycerol, 0.5 mM DTT) followed by the drop-wise addition of an equal volume of hypertonic buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 800 mM KCl, 0.2 mM EDTA, 25% [v/v] glycerol, 0.5 mM DTT, 1% [v/v] NP-40) with constant mixing. Both hypotonic and hypertonic buffers were supplemented with 1 x Complete Mini Protease Inhibitor Cocktail (Roche). Samples were incubated at 4 °C for 45 min on a slowly rotating orbital shaker. Nuclei were collected by centrifugation at 14000 x *g* for 15 min at 4 °C and the nuclear protein fraction (supernatant) aspirated and stored at -80 °C. Protein concentrations of both cytoplasmic and nuclear protein were determined using the Bradford method described in section 4.2.3.

4.2.6 GeneChip target preparation and array hybridization

RNA extractions were used to prepare labelled targets to be used for hybridization to Affymetrix GeneChip expression microarrays. All equipment and kit reagents used in this process were purchased from Affymetrix and used according to the manufacturer's instructions. The composition of all buffers was proprietary information and samples/reagents were retained on ice unless otherwise indicated.

Double-stranded (ds)cDNA was prepared from total RNA extracts. RNA samples (5 µg) were spiked with poly-A RNA controls (1:50000 dilution of stock) using the GeneChip Eukaryotic Poly-A RNA Control Kit. cDNA was synthesised from RNA extracts using a One-Cycle cDNA Synthesis Kit. RNA was mixed with T7-Oligo(dT) Primer (8.3 µM) and incubated for 10 min at 70 °C and for 2 min at 4 °C to allow primer binding. RNA was mixed with 1^{st}-strand synthesis master mix (1^{st} Strand Reaction Mix, 100 mM DTT, 0.5 mM dNTP) and incubated at 42 °C for 2 min. Reactions were mixed with 1 µL SuperScript II, incubated at 42 °C for 1 h and cooled to 4 °C for 2 min. Reactions were mixed with 2^{nd}-strand synthesis master mix (2^{nd} Strand Reaction Mix, 0.23 mM dNTP, DNA ligase, DNA polymerase I, RNase H) and incubated at 16 °C for 2 h. Reactions were mixed with 2 µL T4 DNA polymerase, incubated for 5 min at 16 °C and mixed with EDTA (33 mM). Double-stranded cDNA was cleaned using a GeneChip Sample Cleanup Module kit. cDNA Binding Buffer was mixed with cDNA synthesis reactions, applied to Cleanup Spin Columns and

centrifuged at 8000 x *g* for 1 min. Columns were washed with cDNA Wash Buffer. The column membranes were dried by centrifugation at 25000 x *g* for 5 min with the column caps left open. cDNA was eluted in 14 µL of cDNA Elution Buffer.

Single-stranded biotinylated cRNA was synthesised from cDNA by *in vitro* transcription (IVT) using a GeneChip IVT Labeling Kit. cDNA was mixed with IVT labelling master mix (IVT labelling buffer, IVT labelling NTP mix, IVT labelling enzyme mix) and incubated at 37 °C for 16 h. Labelled cRNA was cleaned using a GeneChip Sample Cleanup Module kit. IVT reactions were mixed with cRNA Binding Buffer and 35% (v/v) ethanol, applied to cRNA Cleanup Spin Columns and centrifuged at 8000 x *g* for 15 s. Columns were washed with IVT cRNA Wash Buffer and 80% (v/v) ethanol. The column membranes were dried by centrifugation at 25000 x *g* for 5 min with the column caps left open and labelled cRNA eluted in 11 µL RNase-free H_2O. IVT cRNA yields were quantified using a NanoDrop spectrophotometer (Thermo Scientific). Labelled cRNA (20 µg) was mixed with Fragmentation Buffer and incubated at 94 °C for 35 min.

Fragmented, labelled cRNA was hybridized to Rat Expression Set 230 (2.0) GeneChip arrays using Hybridization, Wash & Stain Kits. cRNA (15 µg) was mixed with hybridization master mix (3 nM Control Oligonucleotide B2, Eukaryotic Hybridization Controls, Hybridization Mix, DMSO) and incubated at 99 °C for 5 min and 45 °C for 5 min. GeneChip arrays were incubated with Pre-Hybridization Mix at 45 °C for 10 min. Hybridization mix was applied to individual GeneChip arrays and incubated at 45 °C for 16 h. GeneChip incubations were carried out in an Affymetrix Hybridization Oven 645.

4.2.7 GeneChip washing, staining, scanning & analysis

GeneChip washing and staining were performed using an Affymetrix Fluidics Station 450 in conjunction with Hybribization, Wash & Stain Kit reagents. Scanning was performed using an Affymetrix Gene Array Scanner 3000. All of these procedures were set up and controlled using GeneChip Operating Software (GCOS v. 1.4). Hybridized arrays were removed from the hybridization oven and hybridization mixtures replaced with Wash Buffer A. Staining reagents were prepared by transferring

1.2 mL of Stain Cocktail 1 (containing streptavidin-phycoerythrin; SAPE) into a light-protected 1.5 mL tube and 600 µL of Stain Cocktail 2 (containing biotinylated goat anti-streptavidin IgG) and 800 µL of Array Holding Buffer into clear 1.5 mL tubes. Arrays and staining reagents were inserted into the appropriate fluidics station modules. Arrays were washed sequentially with Wash Buffer A (10 cycles of 2 mixes/cycle) at 30 °C and with Wash Buffer B (6 cycles of 15 mixes/cycle) at 50 °C. Arrays were stained for 5 min with Stain Cocktail 1 at 35 °C and washed with Wash Buffer A (10 cycles of 4 mixes/cycle) at 30 °C. Arrays were stained sequentially with Stain Cocktail 2 and with Stain Cocktail 1 (both at 35 °C for 5 min) and washed with Wash Buffer A (15 cycles of 4 mixes/cycle) at 35 °C. Arrays were filled with Array Holding Buffer and transferred to the array scanner. Arrays were scanned using a solid-state 532 nm laser to detect SAPE fluorescence and generate raw fluorescence DAT files. Fluorescence data from control oligonucleotide B2 probes were used to align grids for array image analysis and generate average probe fluorescence CEL files. Spiked labeling and hybridization control probe data were used for array quality control. CEL files were exported using the Data Transfer Tool (v 1.1.1) and were imported into GeneSpring GX (v. 7.3.1) software (Agilent Technologies) for analysis. GeneChip-Robust Multiarray (GC-RMA) normalization was used to determine relative fold-change differences in gene expression between different arrays.

4.2.8 Semi-quantitative RT-PCR

cDNA was amplified from RNA extracts by one-step RT-PCR and amplicons resolved by agarose gel electrophoresis. RT-PCR reactions were prepared in a C2BSC to reduce the risk of contamination. RNA (50 ng) was mixed with Brilliant II RT-PCR master mix (Agilent Technologies), gene-specific forward and reverse primer pairs (0.5 µM each) and AffinityScript RT/RNase block enzyme mixture (Agilent Technologies) to a final reaction volume of 25 µL. Control reactions without RNA template or RT/RNase block enzyme were also prepared. RT-PCR was performed using a Techne Thermocycler (Bibby Scientific) running a thermocycling program consisting of 30 min at 50 °C and 10 min at 95 °C, followed by 35 cycles of 30s at 95 °C, 1 minute at 60 °C and 30 s at 72 °C. Reactions were then mixed with 5 µL of 6 x Gel Loading Buffer (0.05% w/v bromophenol blue, 40% w/v sucrose, 0.1 M pH 8 EDTA, 0.5% w/v SDS)

and resolved by agarose gel electrophoresis as described in section 2.2.10. Gels were visualized using a Molecular Imager FX system (Bio-Rad) set to detect UV fluorescence.

4.2.9 qRT-PCR

qRT-PCR was performed using Brilliant III Ultra-Fast SYBR Green qRT-PCR Master Mix kits (Agilent Technologies) according to the manufacturer's instructions. Reactions were prepared in a C2BSC to reduce the risk of contamination. RNA (25 ng) was mixed with SYBR Green qRT-PCR master mix, gene-specific forward and reverse primers (using previously optimized primer concentrations; see next section), 1 mM DTT, 30 nM ROX reference dye and RT/RNase block enzyme mixture to a final reaction volume of 25 µL. Reactions were performed in 96-well plate format using an Mx3000P system and associated v.2 software (Stratagene) set to detect SYBR1 and ROX fluorescence. The qRT-PCR thermal cycling program comprised 10 min at 50 °C, 3 min at 95 °C and 40 cycles of 95 °C for 20 s and 60 °C for 20 s. Fluorescence was measured at the 60 °C step of each amplification cycle and amplification curves recorded. DNA melt curves were generated by cooling reactions to 55 °C and increasing the temperature to 95 °C over 30 min with fluorescence measured every 20 s. SYBR1 fluorescence was normalized to ROX fluorescence and the SYBR1 amplification curves were used by the software to generate Ct values utilizing adaptive baseline and amplification-based threshold algorithm enhancements. Ct values were used to calculate relative fold-changes in gene expression between control and experimental samples by the $2^{-\Delta\Delta Ct}$ method (described by Livak & Schmittgen, 2001). Briefly, all sample Ct values obtained from relevant genes were normalized to Ct values produced by the normaliser gene *rps23* (coding for a component of the 40S ribosomal subunit) for each individual sample. Normalized Ct values were used to determine relative fold-changes in gene expression. Each qRT-PCR reaction plate included no-template and no-reverse transcriptase controls for each gene-specific master mix employed. Each sample was analysed in duplicate on each plate, and each experimental plate was duplicated.

4.2.10 qRT-PCR optimization and validation

Accurate calculation of specific gene expression by qRT-PCR and the $2^{-\Delta\Delta Ct}$ method requires that the amplification efficiency of the normaliser gene (*rps23*) must be comparable to that of the gene under analysis. Optimal primer concentration of each gene-specific primer pair was determined by RT-PCR using the reagents, equipment and thermocycling conditions described in the previous section. Primer concentrations of 100, 200, 500 and 900 nM were tested for each individual forward and reverse primer. Ct and dRN Last (final fluorescence) values were recorded for the amplification curves produced by each primer pair tested and primer concentrations producing the lowest Ct and highest dRN Last values chosen for further use. An optimal concentration of 500 nM was determined for all primers tested, with the exception of the *Defa24* reverse primer, which had an optimal concentration of 900 nM. Amplification efficiencies of RT-PCR reactions were determined by qRT-PCR using different quantities of template reference RNA ranging from 100-0.01 ng. Reference RNA was obtained by mixing equimolar volumes of all experimental and control RNA extractions (n=288). Ct values of reactions using different initial template quantities were used to construct standard curves and calculate amplification efficiencies (Figure 4.3). The average amplification efficiency of *rps23* was ~103%. Amplification efficiency between 90-110% was deemed acceptable for valid quantification of relative gene expression by qRT-PCR. Reaction specificity was validated by DNA melt-curve analysis and by amplicon cleanup and sequencing, as described in section 2.2.11.

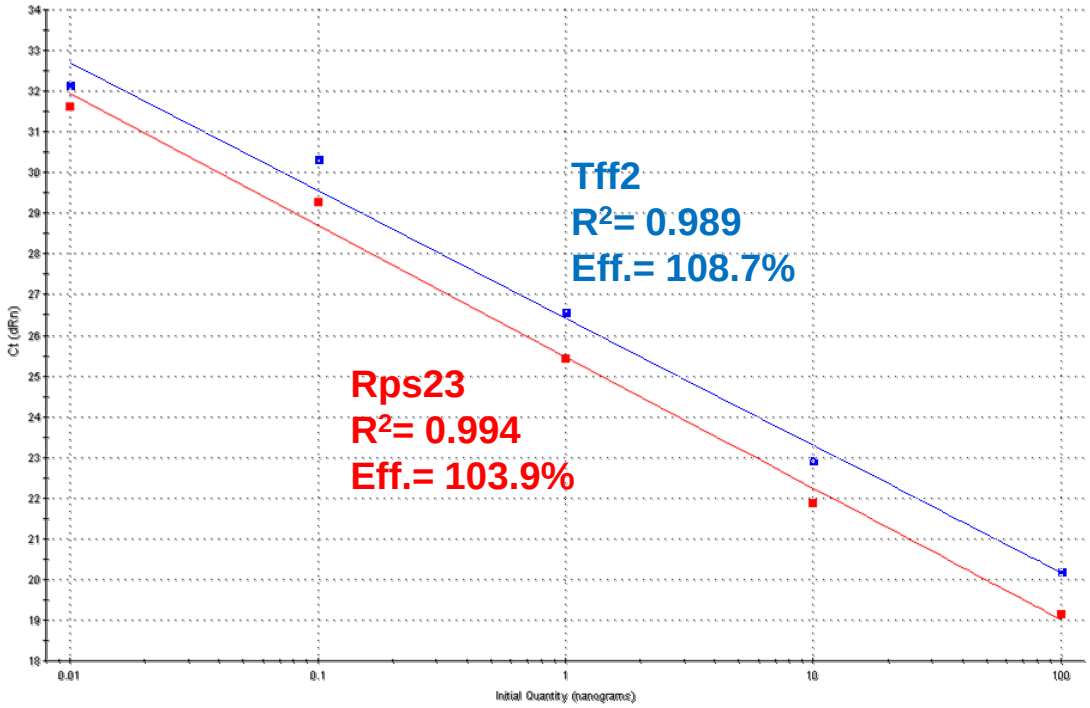


Figure 4.3: *Standard curves utilized to calculate RT-PCR amplification efficiency. Representative plots of normalized Ct values (y-axis) and initial template reference RNA quantity (ng; x-axis) from RT-PCR of rps23 (red) and Tff2 (blue) are illustrated. Pearson correlation coefficients (R^2) and amplification efficiencies (Eff.) are indicated.*

4.2.11 Primary antibody biotinylation

Primary antibodies were biotinylated for use in protein immunodetection methods. Biotinylation of individual IgG antibodies was performed using EZ-Link Sulfo-NHS-LC-Biotinylation Kit reagents (Thermo Scientific) according to the manufacturer's instructions. Antibody solutions were desalted and exchanged into sterile PBS using Zeba Desalt Spin Columns. Columns were equilibrated by three washes of PBS and centrifugation at 1000 x *g* for 2 min. IgG (1 mg) was applied to the column resin and left until fully absorbed. Desalted IgG was eluted by another centrifugation using identical parameters. IgG was mixed with a 20-fold molar excess of Sulfo-NHS-LC-Biotin and incubated on ice for 2 h. Non-conjugated biotin was removed using the Zeba Desalt Spin Column procedure and biotin-conjugated IgG exchanged into fresh PBS. Biotin incorporation was assessed using a 4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay in cuvette format. The OD_{500} of HABA/avidin solution

was determined using a Lambda 25 spectrophotometer (Perkin-Elmer). Biotinylated IgG was added, mixed with the HABA/avidin solution and OD_{500} measured. The decrease in OD_{500} observed after addition of biotinylated IgG was used to calculate moles of incorporated biotin per mole of protein using a HABA assay calculator (http://www.piercenet .com/haba/). Calculated values typically ranged from 5-9 moles biotin/mole IgG.

4.2.12 Tff2 competitive-ELISA

Competitive ELISA was used to quantify Tff2 protein in denatured tissue protein extractions. Goat polyclonal anti-Tff2 primary antibody (sc-23558; Santa Cruz Biotechnology) was biotinylated and diluted 1:100 in filter-sterilized (0.22 µm MILLEX GP; Millipore) blocking buffer (1 % [w/v] molecular grade casein in Tris-buffered saline [TBS]) and dispensed in 100 µL aliquots to 0.5 mL microcentrifuge tubes. Protein samples (10 µg) were added to individual tubes containing anti-Tff2 IgG. Recombinant human Tff2 (rhTff2; Sigma Aldrich) was diluted to 15 ng/mL in PBS. This stock was serially diluted twofold and 100 µL of each dilution was added to individual tubes to give a final standard range of 1500-23.4 pg rhTff2. Control tubes containing only anti-Tff2 IgG were also prepared. Standard/antibody, sample/antibody and control tubes were incubated for 6 h at room temperature on a rotating orbital shaker.

rhTff2 solution was diluted to 1 µg/mL in bicarbonate/carbonate coating buffer (100 mM Na_2CO_3, 100 mM $NaHCO_3$ pH 9.6) and 100 µL aliquots transferred into each well of a 96-well Maxisorp Immunoplate (Nunc). Control wells containing only coating buffer were also prepared. Plates were incubated at room temperature with rotation on an orbital shaker for 2 h. Coating solutions were aspirated and wells washed twice with wash buffer (0.05% [v/v] Tween20 in PBS). Wells were blocked with 350 µL of blocking buffer and plates incubated for 2 h under the same conditions. Blocking buffer was aspirated and wells washed twice with wash buffer. Standard/antibody, sample/antibody and control solutions were applied to individual wells and plates incubated for 16 h at room temperature. Antibody solutions were aspirated and wells washed four times with wash buffer. HRP-streptavidin conjugate (Vector Labs) was

diluted to 5 µg/mL in PBS, 100 µL applied to each well and the plates incubated for 1 h. HRP-streptavidin was removed and wells washed four times with wash buffer. Plates were developed by addition of 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate and incubated in the dark for ~5 min. Colour development was terminated by the addition of 100 µL of 0.4 M sulphuric acid. OD_{450} of individual wells was measured using a SPECTROstar Omega plate-reader (BMG Labtech) with wavelength correction set at 570 nm. OD_{450} measurements from standard wells were used to construct standard curves (Figure 4.4) which allowed the quantification of Tff2 from experimental protein samples. All standard, sample and control wells were run in triplicate on each plate, and each plate was duplicated.

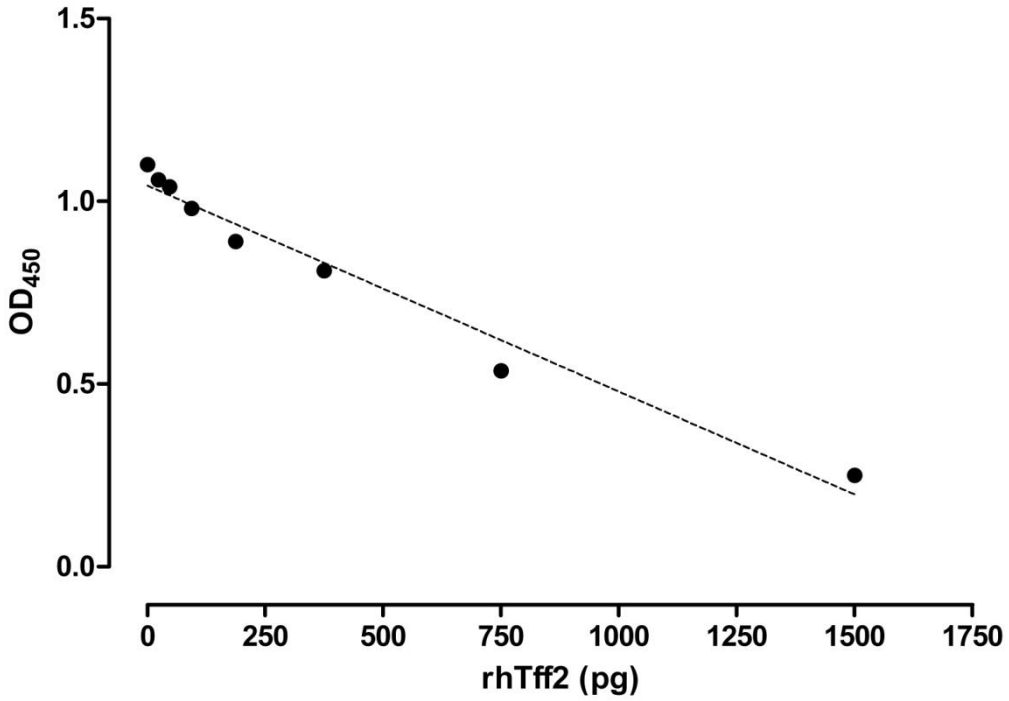


Figure 4.4: *Representative standard curve generated by rhTff2 standards in a competitive ELISA system. Data are mean OD_{450} plotted against the amount of rhTff2 incubated with anti-Tff2 IgG. Line of best fit is illustrated (----).*

4.2.13 Serum cytokine ELISA

IL-6 and IL-1β were quantified from serum samples by sandwich ELISA. All buffers were filter-sterilized using 0.22 µm MILLEX GP filters (Millipore). Blood samples (~200 µL) were obtained from culled neonatal rats and mixed with 200 µL PBS. Serum was obtained by centrifugation of blood at 1500 x *g* for 10 min. Total serum protein was quantified using the Bradford assay and serum stored at -80 °C. IL-6 and IL-1β were quantified using Rat IL-6 or IL-1β ELISA Development kit (PeproTech) reagents according to the manufacturer's instructions. ELISA plates were prepared by coating the wells of 96-well Maxisorp Immunoplates with goat anti-Rat IL-6 (100 ng) and rabbit anti-Rat IL-1β (200 ng) capture antibodies for 16 h. Control wells containing only PBS were also prepared. Wells were washed four times with wash buffer (0.05% v/v Tween20 in PBS), blocked with 350 µL blocking buffer (1% w/v BSA in PBS) for 1 h and washed four times. Standards were prepared by dilution of recombinant rat IL-6 and IL-1β to concentrations of 5 ng/mL and 3 ng/mL respectively in diluent buffer (0.05% v/v Tween20, 0.1% w/v BSA in PBS). Standards were serially diluted twofold and 100 µL of each dilution wells. Serum was diluted in diluent buffer and 100 µg serum protein applied to ELISA plate wells. Control wells containing diluent buffer only were also prepared. Plates were incubated for 2 h and washed four times with wash buffer. Biotinylated goat anti-Rat IL-6 (25 ng) and rabbit anti-Rat IL-1β (50 ng) detection antibodies were applied to wells, incubated for 2 h and wells were washed four times with wash buffer. ELISA plates were developed and OD_{450} measured as described in the previous section.

4.2.14 NFκB electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA; Garner & Revzin, 1981) was used to determine the presence of active (DNA-binding) NFκB transcription factor in nuclear protein extracts. Double-stranded wild-type 5' Cy5-labelled probe and double-stranded wild-type and mutant competitor probe oligonucleotides were prepared by mixing equimolar volumes of complimentary sense and antisense single-stranded oligonucleotides. Mixtures were incubated at room temperature for 10 min to allow strand annealing. Binding reactions were prepared by combining, in order and on ice, 10

µL ddH_2O, 1 µL of poly-deoxyinosinic deoxycytidylic acid (poly-dIdC; 1 mg/mL), 3 µL of 5 x binding buffer (50 mM Tris-HCl, 750 mM KCl, 2.5 mM EDTA, 0.5% [v/v] Triton X-100, 62.5% [v/v] glycerol, 1 mM DTT), 5 µL of nuclear protein extract (5 µg total protein) and 1 µL of labelled probe (10 ng/mL). For competitor reactions, 1 µL of non-labelled wild-type or mutant competitor oligonucleotides (1 µg/mL) was added to binding reactions immediately prior to Cy5-labelled probe. Reactions were incubated for 30 min. Non-denaturing 5% (w/v) polyacrylamide gels (1 mm thick) were prepared using 30% (w/v) acrylamide/bis-acrylamide solution (Bio-Rad), Tris-Borate-EDTA buffer (TBE; 0.89 M Tris-borate, 20 mM EDTA pH 8.3), ddH_2O, 10 % (w/v) ammonium persulphate (APS) and tetramethylethylenediamine (TEMED). Gel mixes were cast using Bio-Rad mini gel-casting apparatus. Set gels were loaded into mini-Protean gel electrophoresis modules (Bio-Rad) and the apparatus filled with 0.5 x TBE buffer. Binding reactions were loaded onto polyacrylamide gels and resolved by polyacrylamide gel electrophoresis (PAGE) at a constant 10 mA current. EMSA PAGE was conducted at an ambient temperature of 4 °C. EMSA gels were scanned using a Molecular Imager FX system (Bio-Rad) set to detect Cy5 fluorescence.

4.2.15 SDS-PAGE

Sodium dodecyl sulphate (SDS)-PAGE was used to resolve both nuclear protein extracts and denatured protein extracts. PAGE was performed using 10% or 5% (w/v) polyacrylamide resolving gels. Protein extracts were diluted to desired concentrations in 10 µL PBS and combined with an equal volume of 2 x Laemmli Sample Buffer (4% w/v SDS, 20% w/v glycerol, 10% v/v β-mercaptoethanol, 0.004% v/v bromophenol blue, 125 mM Tris-HCl pH 7). Proteins were denatured by heating to 95°C for 5 min. Resolving gels were overlaid with a 4% (w/v) polyacrylamide stacking gel, whole gels loaded into mini-Protean gel electrophoresis modules (Bio-Rad) and the apparatus filled with electrode buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS). Protein samples were loaded into stacking gels and separated by electrophoresis at 120 V until the bromophenol blue dye-front reached the bottom of the gel. PAGE gels were washed in ddH_2O and used in Western blots or stained for total protein using Coomassie stain (0.25% [w/v] Coomassie Blue, 10% [v/v] acetic acid, 40% [v/v] methanol). Non-protein bound Coomassie stain was removed using destaining solution (10 % [v/v] acetic acid,

40 % [v/v] methanol) and stained proteins visualised by scanning the gels with a Molecular Imager FX system (Bio-Rad) set to detect Coomassie Blue.

4.2.16 Western blots

Western blots were used to detect Muc2 in denatured protein extracts and α-Tubulin in nuclear protein extracts. Protein was transferred from SDS-PAGE gels to Immobilon-P PVDF membranes (0.45 µm pore size; Millipore) using a Mini Trans-Blot Cell (Bio-Rad). Transfers were performed at a constant voltage of 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine). Membranes were blocked using filter-sterilized (0.22 µm MILLEX GP; Millipore) 1% (w/v) BSA in TBS for 1 h and rinsed twice in wash solution (0.05% Tween20 in TBS). Primary antibody was diluted in diluent solution (0.1% w/v BSA in TBS) and applied to membranes. The primary antibody used to detect α-Tubulin was rabbit polyclonal anti-α-Tubulin IgG (ab4074; Abcam) at a dilution of 1:500. Muc2 was detected using rabbit polyclonal anti-Muc2 IgG (sc-15334; Santa Cruz Biotechnology) at a dilution of 1:500. Muc2 antibody was biotinylated before use as described in section 4.2.11. Membranes were incubated with primary antibody for 16 h at 4 °C. Primary antibody solutions were removed, membranes rinsed and washed four times for 2 min in wash solution. Secondary detection reagents were diluted in diluent solution and applied to membranes. Anti-α-Tubulin IgG was detected using goat anti-rabbit IgG conjugated to AlexaFluor 546 fluorophore (Invitrogen) at a dilution of 1:1000 and anti-Muc2 IgG was detected using HRP-streptavidin conjugate (Vector Labs) diluted to 5 µg/mL. Membranes were incubated with secondary detection reagents for 1 h, rinsed and washed four times in wash solution. Membranes stained to detect α-Tubulin were scanned using a Molecular Imager FX system (Bio-Rad) set to detect AlexaFluor 546. Membranes stained to detect Muc2 were developed by application of TMB Liquid Substrate and photographed.

4.3 Results

4.3.1 Development of P2-P9 gastrointestinal tract tissues

The growth of neonatal rat intestinal tissue was observed over the P2-P9 period, during which susceptibility to systemic *E. coli* K1 infection was lost. Two litters were used. Whole intestinal and gastric tissues were removed each day from two neonates and the length of the small intestine (duodenum-caecum) and colon (caecum-rectum) recorded (Figure 4.5). Representative whole tissues (stomach-colon) were aligned and photographed (Figure 4.6).

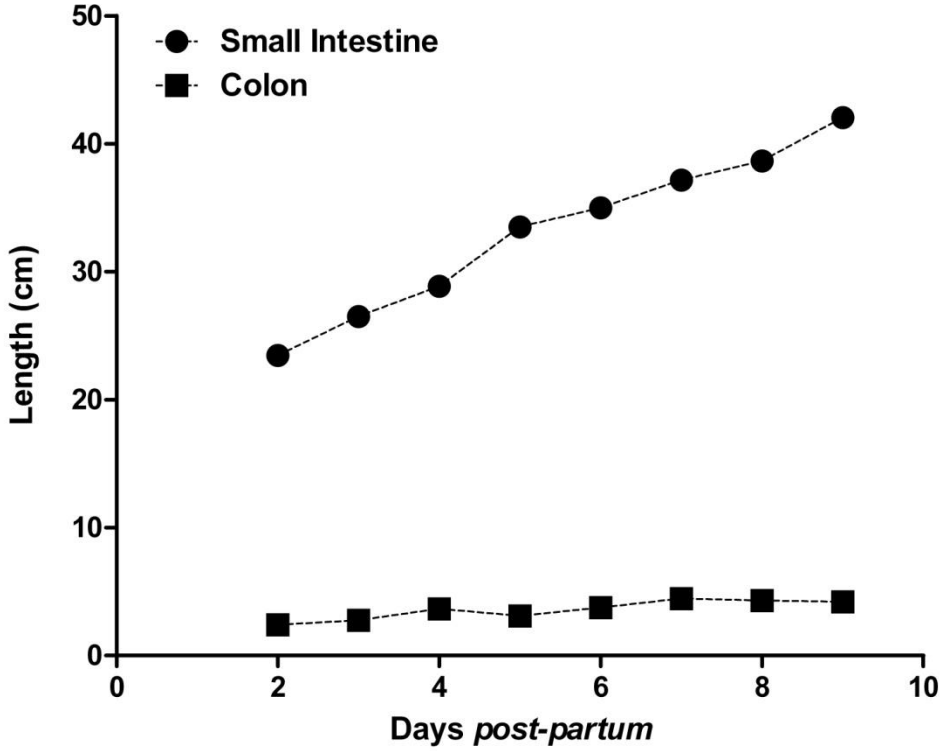


Figure 4.5: *Metrics of neonatal intestinal development. Whole intestinal tissues were removed from P2-P9 neonates and the small intestine and colon measured. Data points are average length of two samples.*

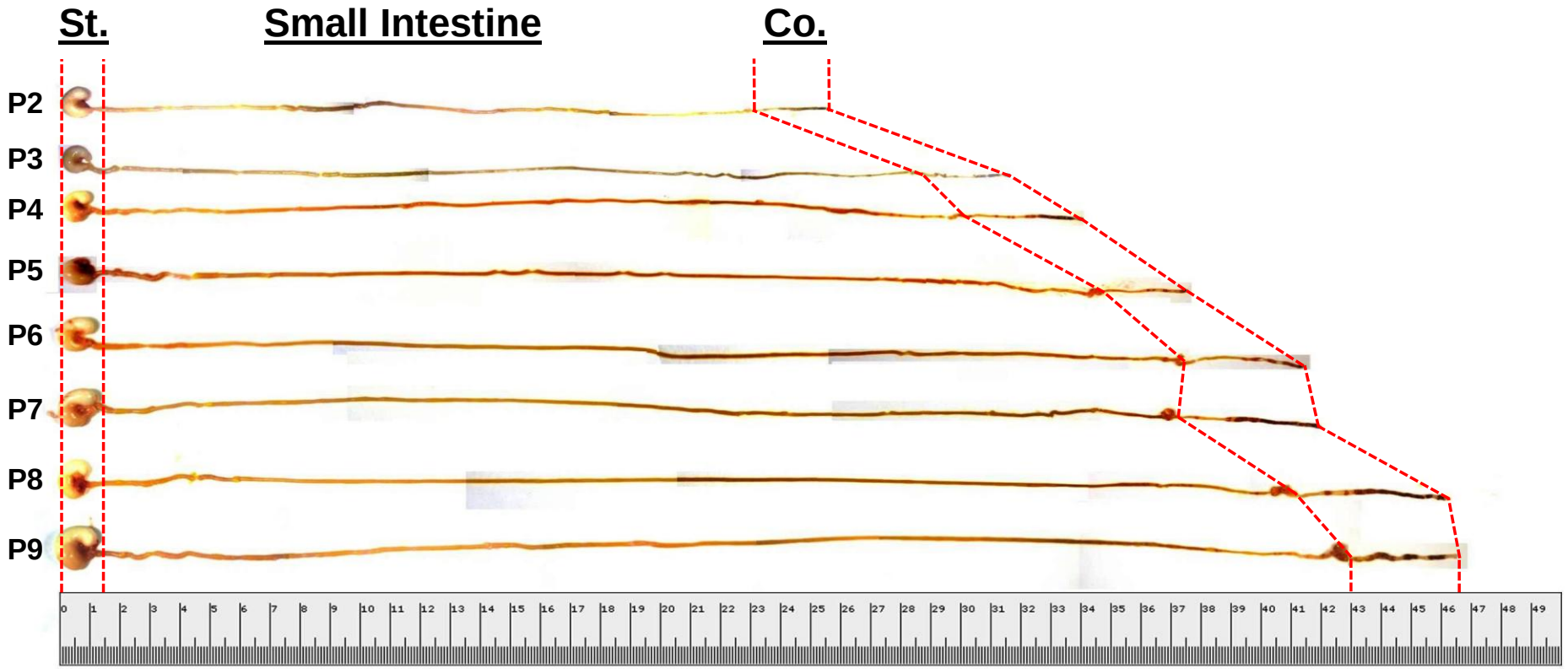


Figure 4.6: *Development of the neonatal rat intestine. Whole intestinal tract tissues were removed from P2-P9 neonatal rats and photographed. Regions comprising the stomach (St.), small intestine and colon (Co.) are indicated.*

The neonatal intestine increased in length by almost 80%; this increase was due predominantly to expansion of the small intestinal tissues. The small intestine increased in length on average by 2.6 cm/day and the colon by 0.25 cm/day. The proportion of the intestine comprised by each element remained approximately constant (90% small intestine, 10% colon) over this period. The caecum was larger in older neonates compared to younger animals. Thus, there was a substantial degree of macroscopic tissue development over the P2-P9 period.

4.3.2 Intestinal tissue transcriptomics

The transcriptome of the neonatal intestine was examined to identify developmentally regulated genes and differential gene expression in response to *E. coli* K1 colonization. RNA was extracted from P2 and P9 intestinal tissues 12 h after colonization with A192PP. RNA was also extracted from non-colonized P2 and P9 intestinal tissues. Five neonates were used in each group. Equimolar volumes of each RNA extract from each group were pooled and each pool hybridized to GeneChip expression microarray. Pooled rather than individual samples were employed due to the limited number of microarrays available. Relative mRNA fold-changes between experimental groups were compared. Differentially expressed genes were assigned to functional categories in a similar fashion to previous investigators (Moen *et al.*, 2008; Zelmer *et al.*, 2010). Gene functions were determined using the DAVID Bioinformatics Resource (v. 6.7; http://david.abcc.ncifcrf.gov/) developed by Huang *et al.* (2009). Genes were assigned to functional groups based on the primary function of their product. Gene products which regulate transcription of functionally diverse genes were assigned to the 'transcriptional regulation' group. Similarly, intracellular signal transducers that mediate diverse signals were assigned to the 'signal transduction' group. Gene products with unknown functions were assigned to the 'unknown' group or 'integral membrane proteins' group where appropriate. All differentially regulated genes are shown in Appendix B.

4.3.2.1 P2-P9 developmental gene expression

Developmental gene expression over the P2-P9 period was assessed by normalization of non-colonized P9 to non-colonized P2 data. Thus, up-regulated genes were those with increased expression at P9 and down-regulated genes were those with increased expression at P2 (Figure 4.7). Substantially more genes showed increased expression at P9 (255 genes) compared to P2 (44 genes).

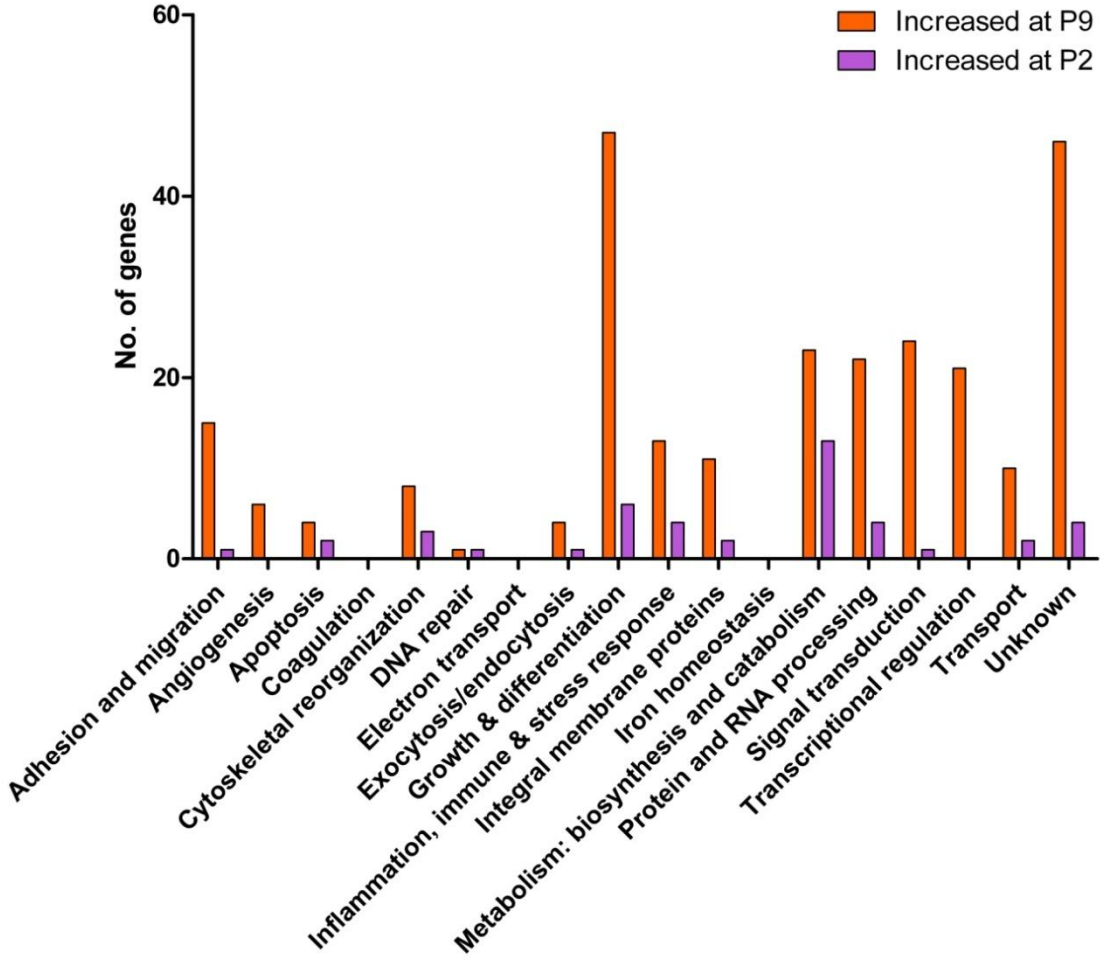


Figure 4.7: *Genes developmentally regulated over the P2-P9 period. Relative gene expression was determined by GeneChip (Affymetrix) microarray analysis of P2 and P9 RNA extracts. Differentially expressed genes were categorized using the DAVID Bioinformatics Resource (v. 6.7; http://david.abcc.ncifcrf.gov/).*

The largest group of differentially regulated genes at P9 were those involved in growth and cellular differentiation. These included genes coding for products involved in epithelial development and the establishment of cellular polarity (*Nr2f2, Fzd3),* as well as genes that stimulate the differentiation of immune cells (*Lrrc8a*, *Sox4*, *Bcl112*). Expression of the pro-inflammatory cytokine gene *Il18* was increased in P2 neonates. The expression of several AMP genes was increased in P9 neonates. These included the α-defensin genes *Defa24* and *Defa-rs1*, as well as phospholipase A2 (*Pla2*) and the putative AMP gene *Dmbt1*. The gene with the highest fold-increase in expression over the P2-P9 period was *RT1-AW2* (21-fold), which encodes a class Ib major histocompatibility complex (MHC Ib). The expression of genes encoding the gel-forming mucins (e.g. Muc2), Tff3 or Fcgbp did not alter over this period. However, expression of *Tff2*, which encodes another member of the trefoil family, was substantially increased (23-fold) in P2 compared to P9 neonates. The decrease in *Tff2* expression over P2-P9 represented the largest decrease observed. Thus, a large number of genes are developmentally regulated in the neonatal intestine over the P2-P9 period, some encoding products that play a role in the defence of host tissues.

4.3.2.2 Response to *E. coli* K1 colonization

The transcriptomic response of neonatal intestinal tissues to *E. coli* K1 colonization was assessed by normalization of colonized P2 and P9 data to equivalent non-colonized data. A substantial number of genes were differentially regulated in both P2 (267 genes; Figure 4.8A) and P9 (617 genes; Figure 4.8B) tissues in response to colonization. However, only thirty of these genes were shared between the P2 and P9 responses (Figure 4.8C). The functional group with the most shared genes was the immune and stress response group. In terms of up-regulated genes, these included *RT1-Aw2* and the C-type lectin AMP *Reg3B*. Down-regulated genes from this group included MHC class I and II genes (*RT1-A3* and *RT1-Db*) and the mast-cell protease gene *Mcpt3*. Other similarities included an up-regulation of *Sox4* and other putative cellular differentiation regulators.

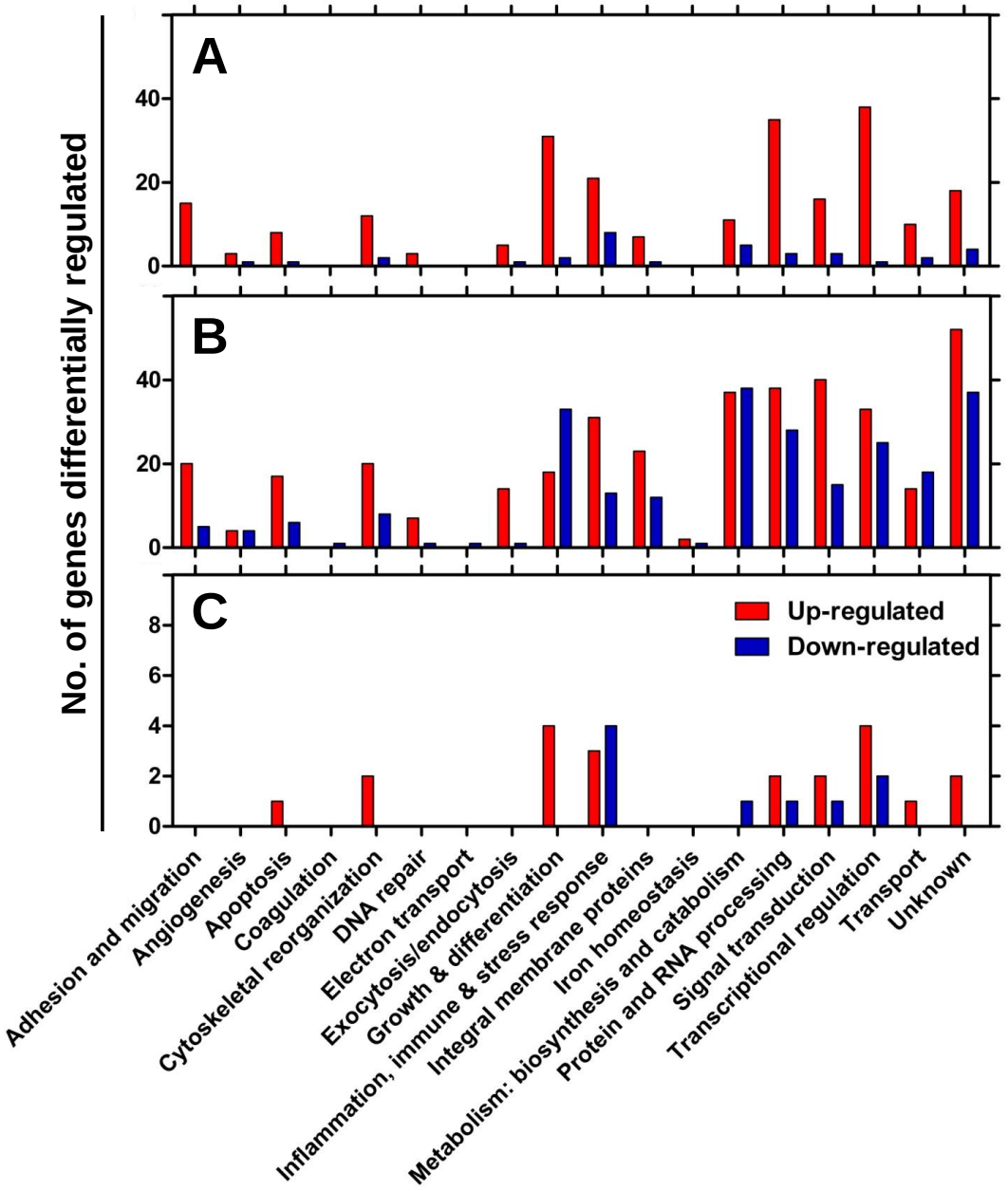


Figure 4.8: *Transcriptomic response of P2 and P9 intestinal tissues to E. coli K1 colonization. Relative gene expression was determined by GeneChip (Affymetrix) microarray analysis of P2 (A) and P9 (B) RNA extracted from neonates colonized by A192PP for 12 h. A subset of genes was shared between the P2 and P9 responses (C). Data from colonized neonates were normalized to data from non-colonized equivalents. Differentially expressed genes were categorized using the DAVID Bioinformatics Resource (v. 6.7; http://david.abcc.ncifcrf.gov/).*

In both neonatal groups, a large number of different genes were up-regulated that were involved in diverse transcriptional regulation, signal transduction, RNA/Protein processing pathways and the regulation of cytoskeletal functions. However, a substantial number of genes belonging to these groups were down-regulated in P9 but not P2 neonates. Genes involved in the expression of several apoptotic initiators and effectors, including *Pdcd4*, *Bid*, *Rtn4*, *Dffb* and the caspase genes *Casp2*, *Casp3* and *Casp8*, were up-regulated in P9 but not P2 neonates. This was accompanied by down-regulation of anti-apoptotic factors, such as *Tgfb2* and *Hspa5*. Pro-apoptotic factor expression was not mirrored in P2 neonates which, conversely, up-regulated the anti-apoptotic mediators *Btg2* and *Iap3*. A number of RT1 genes, which encode the various MHC classes, were differentially regulated in both P2 and P9 neonates (Table 4.3); these include representatives of the MHC I, MHC II and MHC Ib classes. Expression of *RT1-Aw2* increased 17.7-fold in P2 neonates, the largest observed for this cohort. Similarly, *RT1-Bb* expression increased 11.8-fold in P9 neonates. Colonization induced differential expression in several genes encoding components of innate GI defence. In P2 neonates, trefoil factor gene *Tff2* was down-regulated 24.6-fold and in P9 neonates the α-defensin genes *Defa24* and *Defa-rs1* were both up-regulated 3.1- and 5.4-fold respectively, indicating that P2 and P9 neonatal intestinal tissues respond differently to colonization by *E. coli* K1. These differences may influence the capacity of the pathogen to cause systemic disease.

Expression	P2	P9	Shared
Up-regulated	RT1-CE12	RT1-Bb, RT1-CE15	RT1-AW2
Down-regulated	RT1-CE15	RT1-A, RT1-Ba	RT1-Db1, RT1-A3

Table 4.3: *MHC-coding RT1 genes differentially regulated in P2 and P9 neonates in response to E. coli K1 colonization. MHC I (red), MHC II (blue) and MHC Ib (green) classes are indicated.*

4.3.2.3 Microarray validation

Microarray results were validated by qRT-PCR analysis of eleven genes that were differentially regulated in microarray. Genes from both the P2 (*RT1-Aw2, Btg2, Cald1, Tff2* and *Ins2*) and P9 (*Defa-rs1, Pdcd4, Clic4, Cav, Afp* and *Amy2*) datasets were selected for analysis. qRT-PCR data were compared to microarray data and the relationship between the two sets analysed using Pearson correlation (Figure 4.9). The correlation was highly significant (p <0.0001) and demonstrated a good association between microarray and qRT-PCR data.

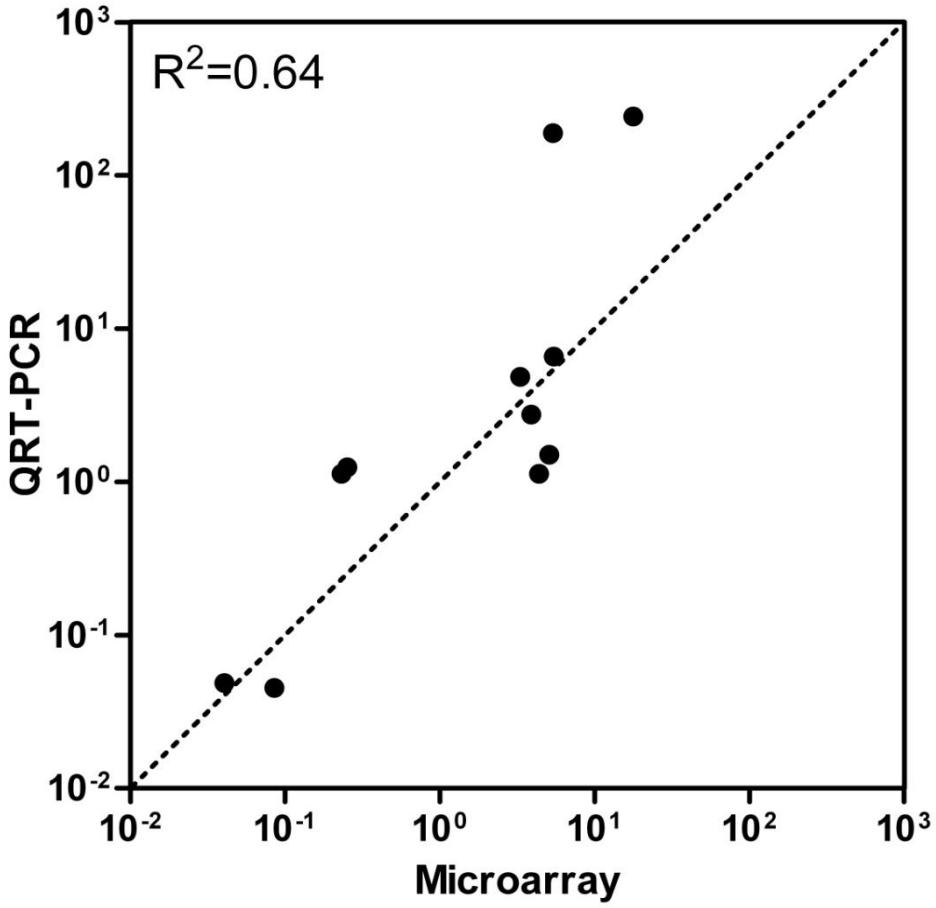


Figure 4.9: *Validation of microarray data using qRT-PCR. The relative expression of 11 genes in E. coli K1-colonized neonates was determined by qRT-PCR of tissue RNA extracts. Mean fold-change in expression detected by qRT-PCR (four replicates) was plotted against equivalent microarray data. The Pearson correlation coefficient (R^2) is indicated.*

4.3.3 Modulation of innate defences by *E. coli* K1

The differential expression of the GI innate defence genes *Tff2, Defa24* and *Defa-rs1* in response to *E. coli* K1 colonization was examined using semi-quantitative and quantitative RT-PCR. The purpose of these experiments was to assess the impact of colonization on expression of these genes over a broader time period in comparison to that examined by microarray. Nine P2 and nine P9 litters (four colonized, four non-colonized [broth-fed] and one control; twelve neonates per litter) were used. RNA was extracted from intestinal tissues of twelve neonates from each colonized and non-colonized group at 6, 12, 24 and 48 h following colonization by strain A192PP. RNA was obtained from the intestinal tissues of three non-inoculated control neonates at these time points.

4.3.3.1 Semi-quantitative analysis

Equimolar volumes of individual RNA samples from each A192PP-colonized and non-colonized group (twelve per group) were pooled and used as templates for RT-PCR. Amplicons were resolved by agarose gel electrophoresis for comparison of non-colonized and colonized groups (Figure 4.10). There was a decrease in *Tff2* expression at 24 and 48 h after colonization in P2 but not P9 neonates. *Defa24* expression was increased at 6 and 12 h after colonization in P9 but not P2 neonates. *Defa-rs1* expression increased in P9 neonates 48 h after colonization. Expression in P2 neonates also increased 24 and 48 h after colonization. Comparison of non-colonized P2 and P9 data indicated that *Tff2* expression decreased and *Defa-rs1* expression increased over the P2-P9 period. No significant difference in expression of the control gene *rps23* was detected between samples. These results did not fully concord with microarray data with respect to the timing of differential gene regulation, possibly due to the fact that they were based on a larger sample size. However, they did indicate that differential regulation of these genes varied over the first 48 h after *E. coli* K1 colonization.

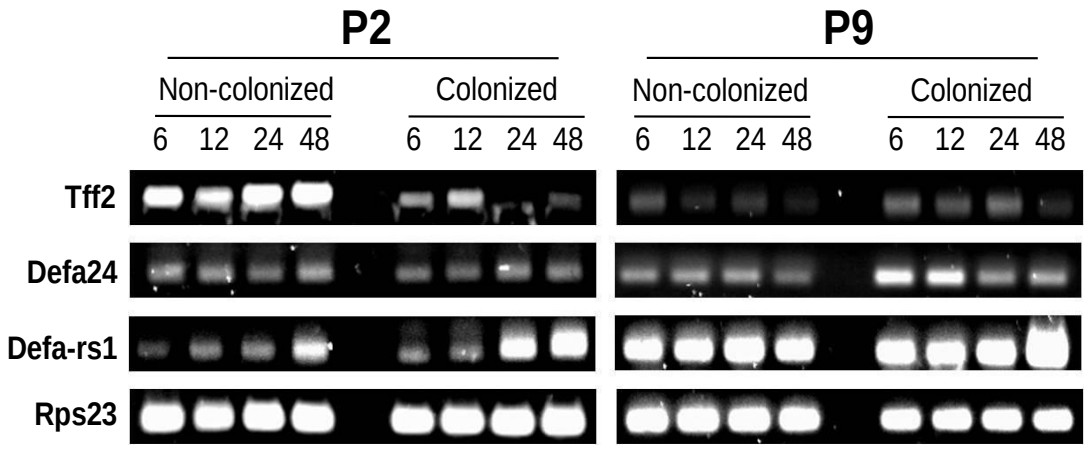


Figure 4.10: *Semi-quantitative RT-PCR analysis of Tff2, Defa24 and Defa-rs1 expression. RNA extracts from A192PP-colonized and non-colonized P2 and P9 neonates were used as templates for RT-PCR amplification of target genes. Amplicons were resolved on 1% (w/v) agarose gels. Rps23 was amplified to serve as a control.*

4.3.3.2 Quantitative analysis

RNA extracts from each experimental group were analysed by qRT-PCR in order to quantify the relative expression of these genes after colonization by *E. coli* K1 (Figure 4.11). Data from colonized and non-colonized neonates were compared by the two-tailed Mann-Whitney test. *Tff2* expression was significantly (p <0.0001) decreased (4.6-fold) in P2 animals at 24 and 48 h after colonization with A192PP. No significant differences in expression of this gene were observed in P9-colonized neonates at any time point examined. *Defa-rs1* expression was increased threefold in P9 neonates (p <0.05) at 6-24 h and 28.5-fold (p <0.0001) 48 h after colonization. Similarly, *Defa24* expression was increased 5.1-fold in P9 neonates at 6 and 12 h after colonization (p <0.001) but not at subsequent time points. No significant differences in either *Defa-rs1* or *Defa24* expression were detected in P2-colonized neonates at any time point examined.

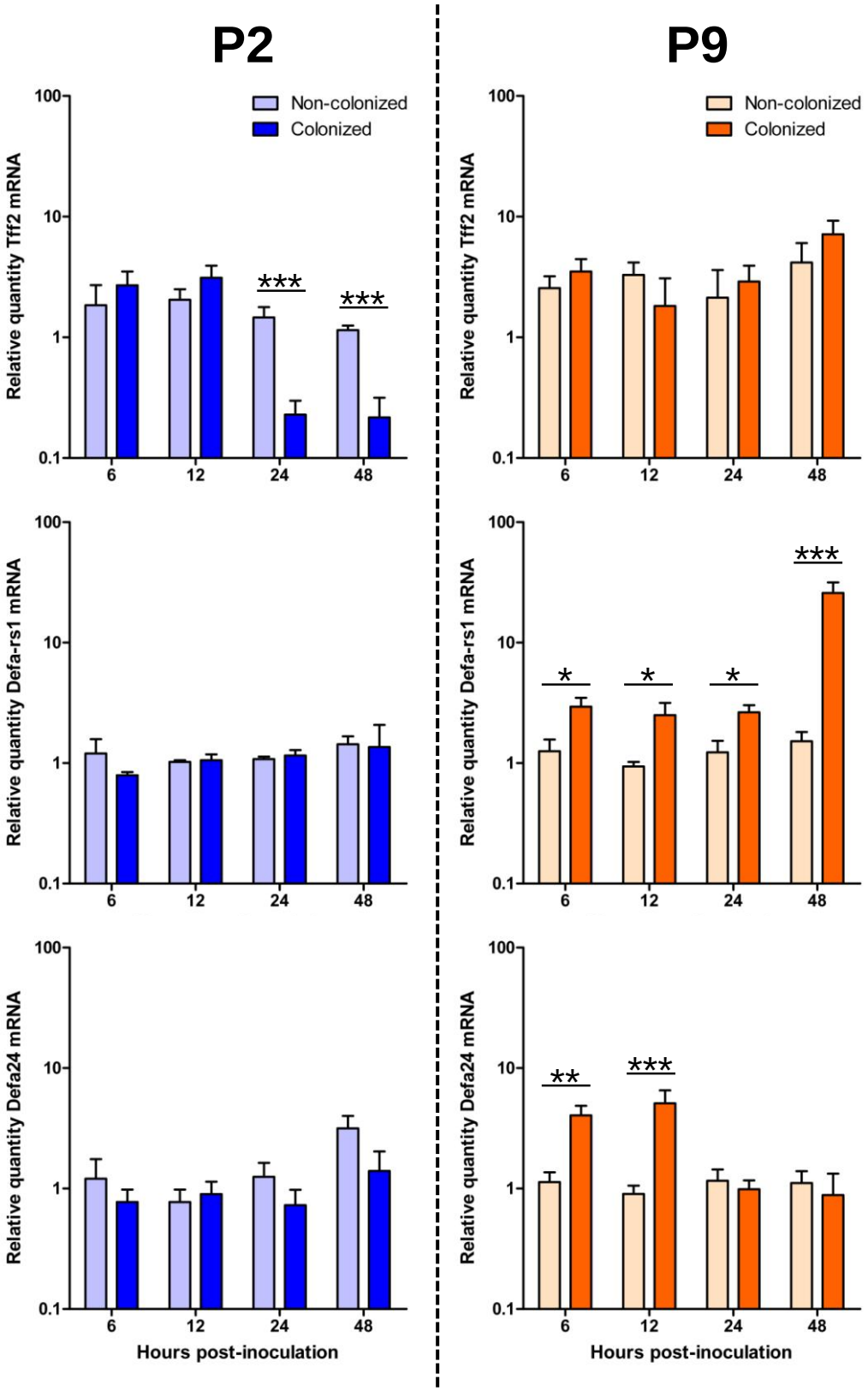

P2
P9
Non-colonized
Colonized
Relative quantity Tff2 mRNA
Relative quantity Defa-rs1 mRNA
Relative quantity Defa24 mRNA
100
10
1
0.1
6
12
24
48
***
*
**
Hours post-inoculation

Figure 4.11: *Quantitative analysis of Tff2 (top), Defa-rs1 (middle) and Defa24 (bottom) expression in P2 and P9 neonates colonized with E. coli K1. RNA was extracted from A192PP-colonized and non-colonized intestinal tissues at the indicated times after colonization and served as a template for qRT-PCR. Data was normalized to mean non-colonized data at each time point. Error bars represent SEM of twelve replicates. Differences between non-colonized and colonized data are indicated; Mann-Whitney (***** $p<0.05$, ****** $p<0.01$, ******* $p<0.001$).*

Tff2 protein was quantified from the intestinal tissues of A192PP-colonized and non-colonized P2 neonates. Protein was extracted from tissues under denaturing conditions at the time points after colonization previously examined. Tff2 protein was quantified by competitive ELISA (Figure 4.12). Results showed a significant (Mann-Whitney; p <0.001) decrease in Tff2 protein at 24 and 48 h after colonization with A192PP. A mean decrease in total Tff2 protein of 3.9- and 2.6-fold was observed at 24 h and 48 h respectively.

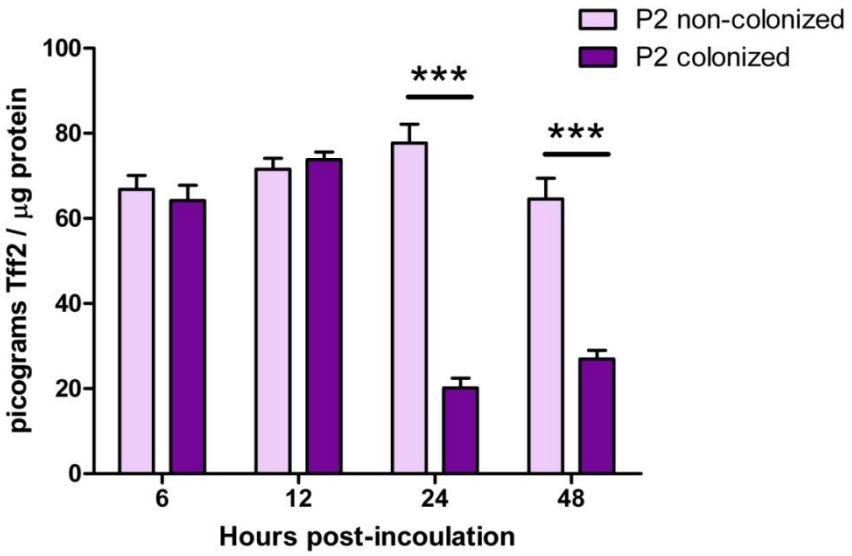


Figure 4.12: *Quantification of Tff2 protein from E. coli K1-colonized and non-colonized P2 intestinal tissues. Protein was extracted from A192PP-colonized and non-colonized P2 neonates at the indicated time points following colonization and concentration determined by competitive ELISA. Data was normalized to total protein concentration. Significant differences between non-colonized and colonized data are indicated; Mann-Whitney (***** $p<0.05$, ****** $p<0.01$, ******* $p<0.001$).*

4.3.3.3 Effect on developmental expression

Transcriptomic analysis indicated that expression of *Tff2, Defa-rs1* and *Defa24* was developmentally modulated over the P2-P9 neonatal period. The 'normal' developmental regulation of these genes was assessed in order to better understand the impact of *E. coli* K1 colonization on their expression. RNA extracts from all non-colonized (broth-fed) neonates were used in this experiment. Intestinal RNA was extracted from non-colonized P1 neonates to serve as a reference. Data from broth-fed neonates was compared to data from control intestinal RNA extracts in order to ensure that feeding of bacteria did not induce changes in gene expression. All samples were analysed by qRT-PCR with data normalized to P1 samples. Data obtained in the previous section were combined with normal expression data in order to demonstrate the effect of A192PP colonization on normal developmental gene expression (Figure 4.13).

Expression of the three genes varied significantly over the P1-P11 developmental period; however, the pattern of regulation differed between *Tff2* and the α-defensin genes. *Tff2* expression increased by 4.5-fold (Mann-Whitney; $p < 0.001$) over P1-P4. The level of *Tff2* expression detected at P4 was maintained until P9, after which expression decreased ($p < 0.001$) to a level fourfold lower than at P1. Colonization with A192PP at P2 reduced expression of *Tff2* to a level similar to that observed at P1. The expression of both α-defensin genes increased substantially from P1-P11. *Defa24* expression increased by 5.8-fold and *Defa-rs1* by 29.2-fold ($p < 0.001$). The increased developmental expression of α-defensin genes amplified the overall up-regulation induced by colonization with A192PP at P9. For example, *Defa-rs1* expression in P9 neonates 48 h after colonization was 704.5-fold greater than expression of this gene at P1.

This data demonstrated that refractive neonates not only increase expression of α-defensins in response to *E. coli* K1 colonization but also express more of these AMPs than susceptible neonates at the time of colonization. Furthermore, the expression of the trefoil factor gene *Tff2* is developmentally regulated in the intestine and *E. coli* K1 colonization disrupts this process in susceptible neonates.

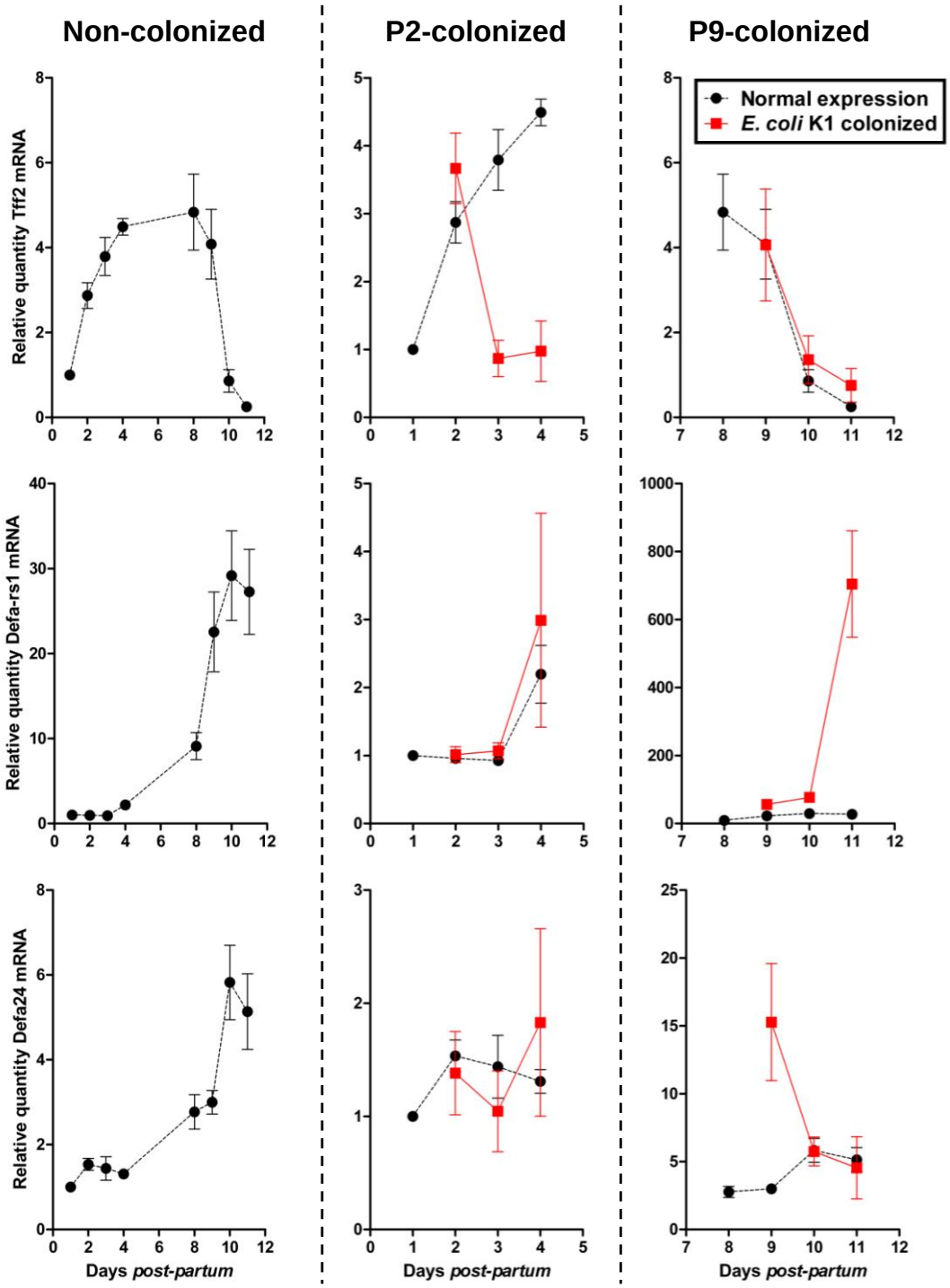


Figure 4.13: *Normal expression of Tff2 (top), Defa-rs1 (middle) and Defa24 (bottom) genes and differential expression induced by E. coli K1 colonization at P2 and P9. Expression was quantified by qRT-PCR of intestinal RNA extracts from non-colonized and A192PP-colonized neonates on the days post-partum indicated. Data normalized to expression of each gene at P1. Error bars represent SEM from at least twelve animals.*

4.3.4 Repression of *Tff2* expression

Trefoil factor expression is modulated by numerous regulatory mechanisms (reviewed by Baus-Loncar & Giraud, 2005). Expression of *Tff2* is negatively modulated by the acute-phase transcriptional regulators nuclear factor kappa B (NFκB) and CCAAT/enhancer-binding protein β (C/EBPβ; Dossinger *et al.*, 2002). These transcriptional repressors are activated by, respectively, IL-1β and IL-6 pro-inflammatory cytokines. Therefore, either of these regulatory mechanisms may be responsible for the decrease in *Tff2* expression observed in susceptible neonates after colonization with *E. coli* K1.

4.3.4.1 IL-6 and IL-1β serum cytokine levels

The release of IL-6 and IL-1β cytokines in response to *E. coli* K1 colonization was assessed by quantification of serum levels. Serum was obtained from P2 and P9 neonates 6, 12, 24 and 48 h after colonization with A192PP. Six animals were used for sampling at each time point and serum was collected from an equal number of age-equivalent non-colonized animals. IL-6 and IL-1β were quantified by ELISA and values obtained from non-colonized and colonized animals compared by Mann-Whitney test (Figure 4.14). Both cytokines were detected in serum of neonates colonized with A192PP at P9; however, no significant differences were detected between non-colonized and colonized neonates at any time point examined. Conversely, only IL-1β was detected in the serum of P2 neonates and significantly (p <0.001) higher levels were detected in animals colonized with A192PP compared to their non-colonized counterparts. Serum IL-1β concentration more than doubled from 6-24 h after colonization in these animals, but returned to non-colonized levels 48 h after colonization. These results show that IL-1β secretion was significantly increased in susceptible neonates in response to *E. coli* K1 colonization.

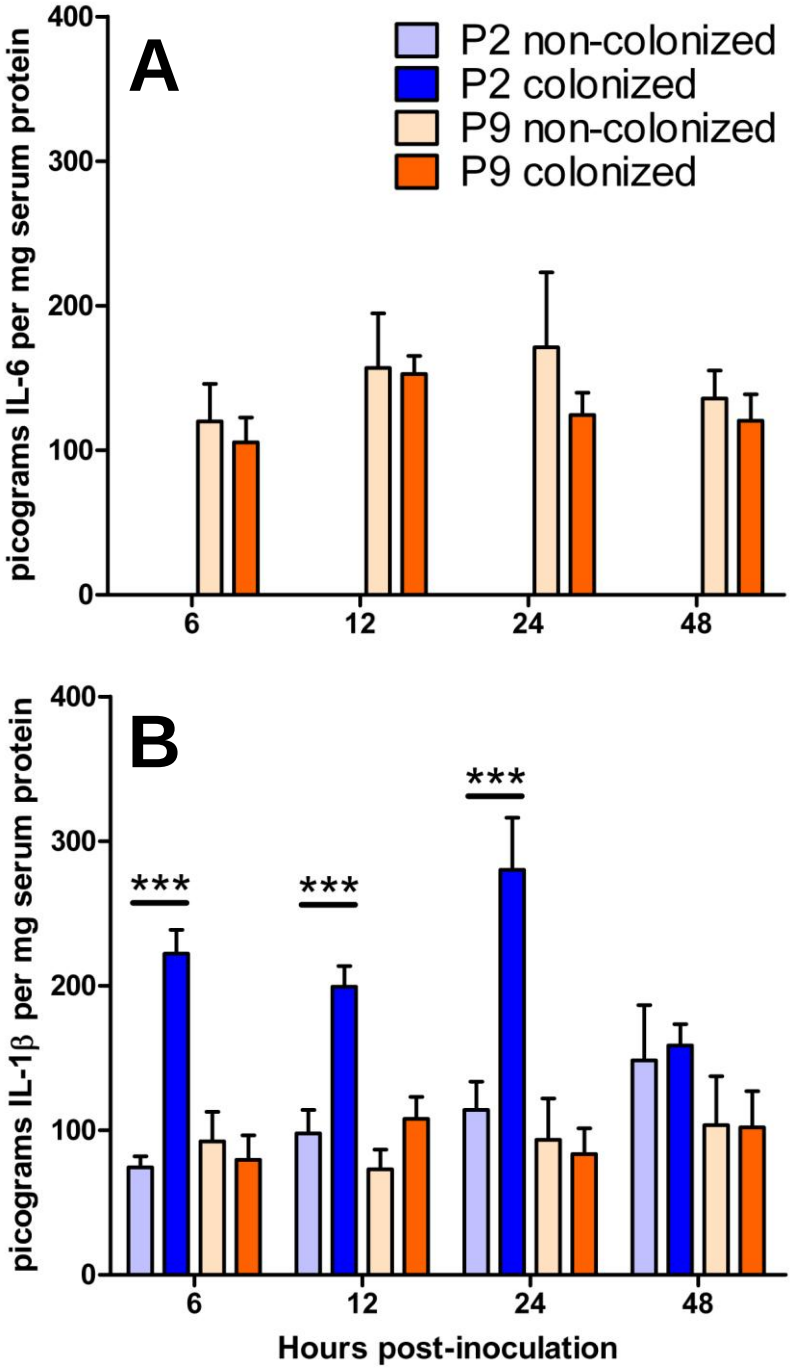


Figure 4.14: *Quantification of IL-6 (A) and IL-1β (B) from neonatal serum. Serum was obtained at the times after colonization indicated from P2 and P9 A192PP-colonized neonates and from age-equivalent non-colonized animals. Error bars represent SEM of six animals. Differences between non-colonized and colonized data are indicated; Mann-Whitney (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).*

4.3.4.2 NFκB and C/EBPβ expression and activity

Expression of the genes encoding NFκB and C/EBPβ was examined to determine if colonization by *E. coli* K1 influenced the production of these transcription factors. The intestinal RNA extracts used in previous gene expression analyses were employed. RNA was examined by qRT-PCR and data from A192PP-colonized animals normalized to data from non-colonized animals (Figure 4.15). Colonization by *E. coli* K1 had no impact on the expression of *Cebpb* at any time point examined. Expression of *Nfkb1* was increased to a small (1.3-fold) but significant (Mann-Whitney; p <0.01) degree 48 h after colonization, providing further evidence that the IL-1β/NFκB pathway was the most likely source of *Tff2* repression. The activity of NFκB was therefore assessed in neonatal intestinal tissues.

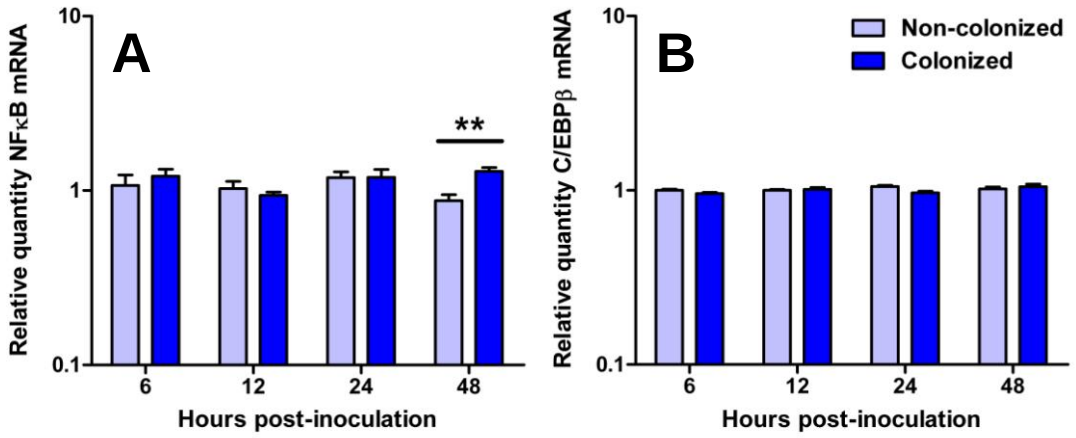


Figure 4.15: *NFκB (A) and C/EBPβ (B) expression in E. coli K1 colonized intestinal tissue. Expression was quantified by qRT-PCR of intestinal RNA from non-colonized and A192PP-colonized P2 neonates at the times indicated. Differences between non-colonized and colonized data are indicated; Mann-Whitney (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).*

NFκB activity was assessed by EMSA of nuclear protein extracts, allowing semi-quantitative assessment of NFκB activity. Single cells were prepared from the intestinal tissues of neonates colonized with A192PP at P2. Tissues were obtained 6, 12, 24 and 48 h after colonization. Nuclear proteins were recovered and examined for cytoplasmic contamination by SDS-PAGE and Western blotting (Figure 4.16).

Comparison of cytoplasmic and nuclear protein fractions by SDS-PAGE showed that nuclear fractions contained intensely-staining low molecular weight (~12-15 kD) protein bands that were largely absent from cytoplasmic fractions. The size of these proteins corresponds to the known molecular weight of several nucleus-associated histone proteins. Furthermore, Western blots detected α-tubulin in cytoplasmic, but not nuclear, protein fractions, demonstrating that nuclear protein was successfully isolated and available for use in downstream EMSA.

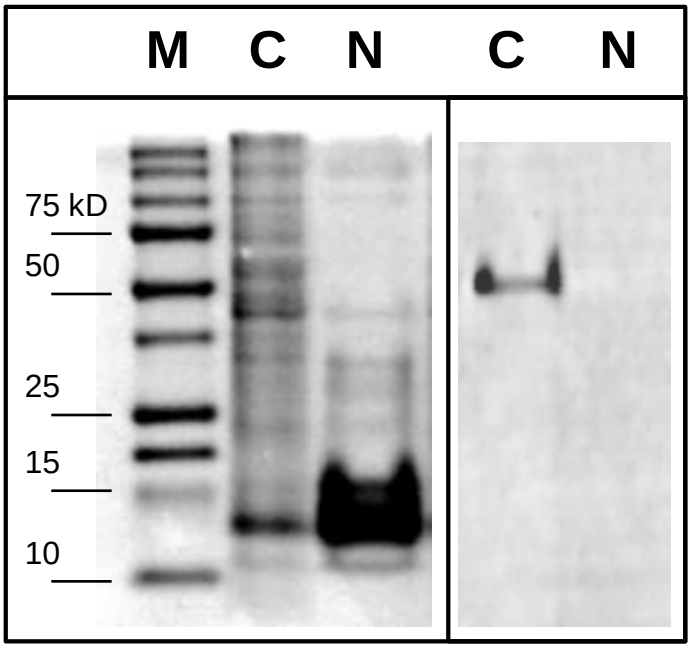


Figure 4.16: *Isolation of nuclear proteins from intestinal tissues. Cytoplasmic (C) and nuclear (N) protein fractions were resolved by SDS-PAGE (left panel) and stained for α-tubulin by Western blot (right panel). Precision-Plus Protein Marker (M; Bio-Rad) was used to determine protein molecular weight.*

Nuclear proteins were obtained from six colonized and non-colonized neonates for each time point examined. Equal amounts of protein extract from individual experimental groups were pooled and analysed by EMSA (Figure 4.17). Assay specificity was checked by competition EMSA analysis of combined pooled nuclear protein extracts. Band shift was detected in all non-colonized and colonized samples at all time points examined. However, the intensity of the band shift was much greater in

colonized samples compared to their non-colonized equivalents. Competitor EMSA showed that the observed band shift was specific to the sequence of the NFκB probe. These results clearly indicated that colonization by *E. coli* K1 significantly increased the amount of active NFκB localized to the nuclei of intestinal tissue cells.

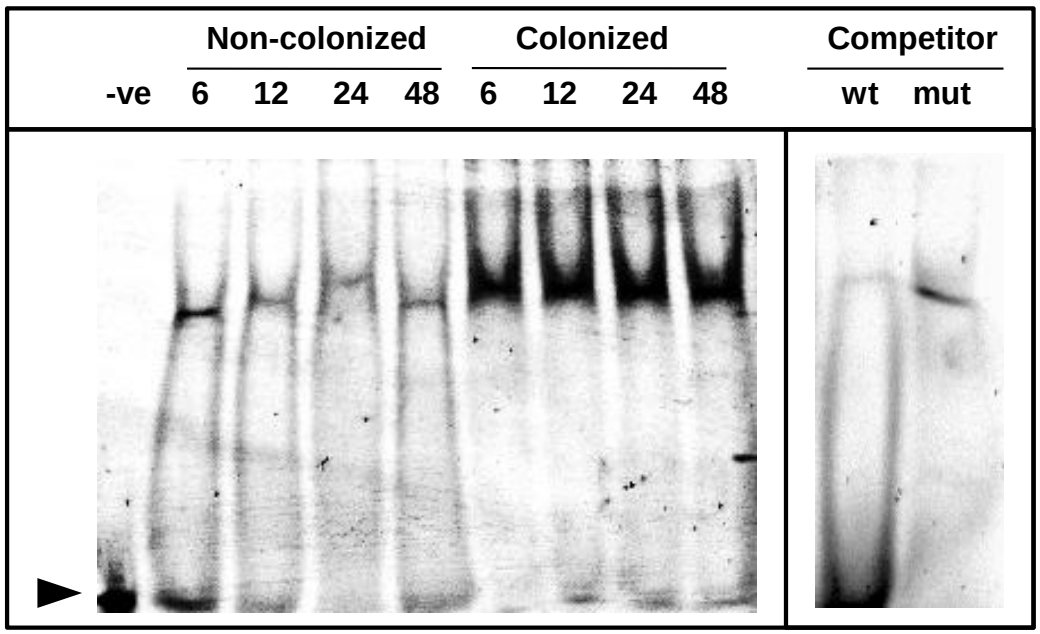


Figure 4.17: *Activation of NFκB by E. coli K1 intestinal colonization. Nuclear protein extracts were obtained from A192PP-colonized and non-colonized P2 neonates at the time points indicated after colonization. Extracts were analysed by EMSA using a Cy5-conjugated dsDNA probe containing the wild-type NFκB binding site (left panel). Competitor EMSA (right panel) was performed using unlabelled wild-type (wt) or mutant (mut) competitor dsDNA. The position of free Cy5-conjugated probe is indicated* (►).

4.3.5 Muc2 expression

Expression of the gel-forming mucin Muc2 was assessed in the intestines of neonates colonized with *E. coli* K1 at P2. Expression was analysed at the mRNA and protein level by qRT-PCR and Western blot. Muc2 expression was quantified from RNA and reduced protein extracts used in previous experiments (Figure 4.18).

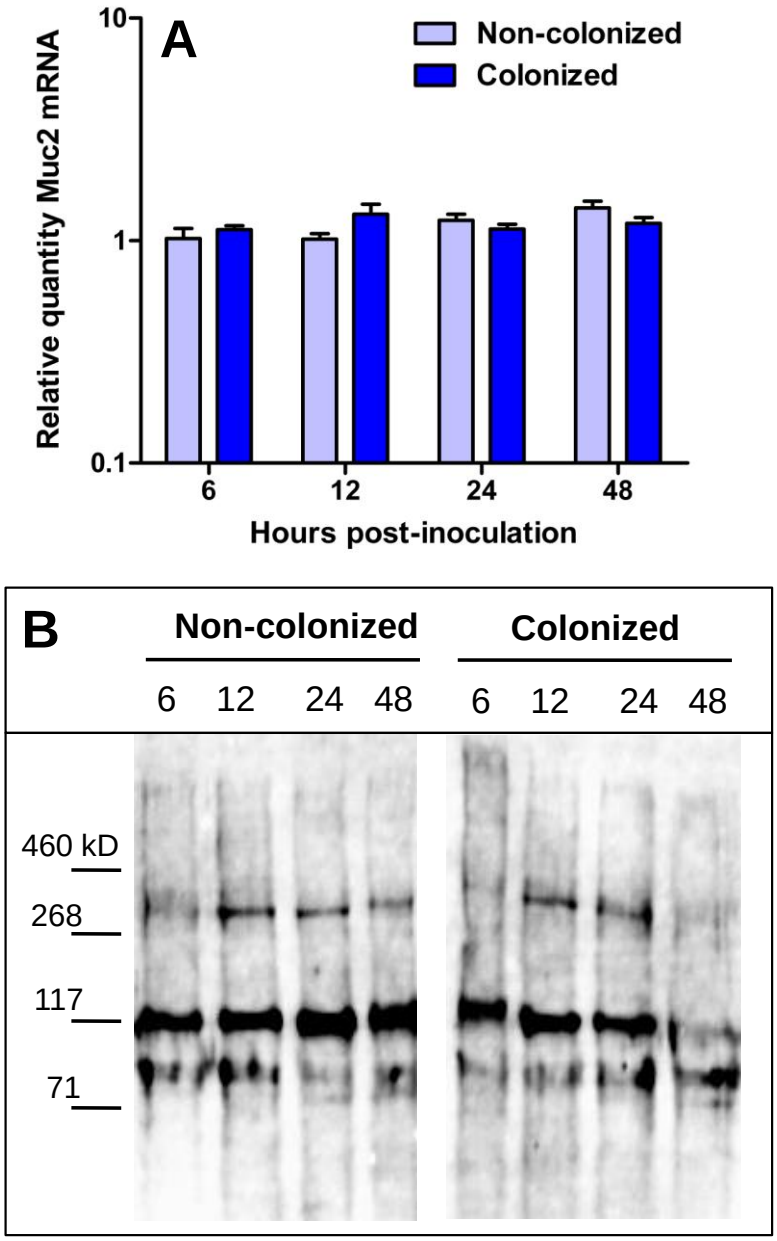


Figure 4.18: *Intestinal Muc2 expression in neonates colonized with E. coli K1 at P2. (A) Expression at the mRNA level was analysed by qRT-PCR of RNA extracts. Error bars represent SEM of twelve replicates. (B) The presence of Muc2 protein was analysed by Western blot of SDS-PAGE resolved reduced protein extracts (n=6; pooled). Samples were obtained from A192PP-colonized and non-colonized neonates at the time points indicated after colonization. Protein band molecular weight was determined using HiMark Protein Standards (Invitrogen).*

No significant differences in *muc2* gene expression between colonized and non-colonized samples were found at any time point between 6-48 h. Western blot analysis detected three immunoreactive protein bands. No bands were detected in control blots using secondary detection reagents only; the reactivity of the bands was therefore anti-Muc2 primary antibody-specific. The approximate molecular weights of these bands were 300, 117 and 90 kD. The 300 kD band represented Muc2 monomer whilst the smaller bands are probably Muc2 degradation products generated during protein extraction. Comparison between A192PP-colonized samples and their non-colonized equivalents showed no significant differences from 6-24 h after colonization. However, after 48 h, both the 300 and 117 kD band were significantly diminished in comparison to the equivalent non-colonized sample. Less Muc2 monomer was detected at the 6 h time point compared to all subsequent time points in both colonized and non-colonized samples. Overall, these results demonstrate that Muc2 protein was reduced by *E. coli* K1 colonization of the neonatal intestine. Furthermore, this reduction was not regulated by the host at the mRNA transcriptional level.

4.4 Discussion

The data presented in this chapter demonstrate that a significant degree of intestinal tissue development occurs at the anatomical and molecular levels over the P2-P9 developmental period. Tissue development occurs over the period during which *E. coli* K1 loses its capacity to translocate from the intestinal lumen to the systemic circulation. It is therefore possible that loss of translocational capacity is related to maturation of the GI tissues.

Comparison of the P2 and P9 transcriptomes indicate that several genes encoding products of the host innate immune system are expressed to a greater degree in the more mature P9 tissues. These include several Paneth cell-secreted AMPs such as phospholipase A2, the enteric α-defensin Defa24, the α-defensin related Defa-rs1 and the putative AMP Dmbt1. Phospholipase A2 is bactericidal to *E. coli* strains and has a similar minimum bactericidal concentration as the human myeloid α-defensin HNP-1 (Harwig *et al.*, 1995). The antimicrobial spectra of Defa24 and Defa-rs1 are currently unknown; however, they are closely related to the murine Defcr (defensin-related cryptidin) and CRS (cryptidin related sequence) enteric α-defensin groups (Patil *et al.*, 2004). The murine α-defensins are well characterized, possess broad antimicrobial activity and are active against *E. coli* (Hornef *et al.*, 2004; reviewed by Ouellette & Selsted, 1996). Dmbt1 is an agglutinin secreted by several cell types including Paneth cells. It is not bactericidal, but binds to and agglutinates many bacterial species, including *E. coli* (Bikker *et al.*, 2002). Dmbt1 inhibits the intracellular invasion of intestinal epithelial cells by *Salmonella enterica* (Rosenstiel *et al.*, 2007). It is therefore possible that some or all of the developmentally regulated AMPs modulate the capacity of *E. coli* K1 to access the intestinal epithelium and cause systemic disease.

The gene with the largest increase in expression in P9 compared to P2 intestinal tissues also encodes an immune-related protein: RT1-Aw2 (also known as RT1-EC2) is an MHC Ib molecule. MHC Ib molecules are very similar to classical MHC Ia molecules in that they are used by nucleated cells to present intracellular material at the cell surface. This material is generally derived from the processing of cytoplasmic proteins by the cytosolic proteasomes and usually consists of normal host peptide

fragments. Presentation of foreign peptides (i.e. during intracellular viral or bacterial infection) or defective host peptides results in activation of cytotoxic T-cells which induce apoptotic pathways in the infected/defective cell. MHC Ia expression is ubiquitous and MHC Ia peptide binding sites are highly polymorphic which allows them to bind a huge range of peptide ligands. Conversely, MHC Ib expression is tissue-specific and their peptide binding sites are oligomorphic and thus bind only a restricted range of ligands. These ligands include specific prokaryotic molecules such as the Hsp60 orthologue GroEL and *N*-formylmethionine, a modified form of methionine which bacteria use to initiate protein synthesis (Colmone & Wang, 2006; reviewed by Rodgers & Cook, 2005). This suggests that one function of MHC Ib molecules is to act as intracellular PRRs that can rapidly present conserved bacterial peptides at the surface of infected cells. This MHC Ib function may be relevant to intracellular *E. coli* K1 infection. Unfortunately, the rat RT1 complex is relatively poorly characterized compared to its human and murine equivalents. It is also very difficult to draw orthologous relationships between MHC molecules based on sequence homology. As such, the ligand specificity and function of RT1-Aw2 remain unknown.

It is clear that substantial development occurs in the intestine over P2-P9. However, it is not clear if any of these alterations are directly responsible for the modulation of susceptibility to *E. coli* K1 infection. The differential responses of P2 and P9 tissues to *E. coli* K1 colonization were assessed in an attempt to shed light on this question. One potentially significant difference was the up-regulation of the developmentally regulated AMPs Defa24 and Defa-rs1. This occurred in P9 but not P2 neonates. Up-regulation of Defa24 and Defa-rs1 at the transcriptional level was unexpected as enteric defensin genes are thought to be constitutively expressed. Bacterial PAMPs can induce increased defensin secretion but not a concomitant up-regulation of defensin mRNA transcription (reviewed by Selsted & Ouellette, 2005). Defa24 expression was up-regulated in response to *E. coli* K1 colonization of P9 neonates; however, this up-regulation was transient and was not detected >24 h after colonization. Conversely, the extent of Defa-rs1 up-regulation increased significantly from 6-48 h after colonization. The regulation of defensin-related peptides has not yet been specifically characterized and the data presented here strongly indicate that expression of this AMP class is inducible. The fact that both Defa24 and Defa-rs1 were up-regulated in response to *E. coli* K1 colonization of P9 but not P2 neonates is

indicative that these two AMPs play a role in modulating susceptibility to the pathogen. A further AMP which was up-regulated in response to colonization was Reg3b. This protein has recently been implicated in the control of Gram-negative bacteria in the intestine (van Ampting *et al.*, 2012). However, this AMP was up-regulated in both P2 and P9 neonates, suggesting that it is unlikely to be of significance in terms of *E. coli* K1 infection.

Colonization with *E. coli* K1 induced differential regulation of multiple MHC genes in P2 and P9 neonates. In both groups, the MHC Ib gene RT1-Aw2 was strongly up- and RT1-Db1 (MHC II) and RT1-A3 (MHC I) down-regulated. RT1-Bb (MHC II) and RT1-CE15 (MHC I) were up-regulated and RT1-Ba (MHC II) and RT1-A (MHC I) were down-regulatde in P9 intestinal tissues. Conversely, RT1-CE15 was down-regulated and RT1-CE12 (MHC I) was up-regulated in P2 intesintal tissues. MHC I genes are regulated by multiple factors including NFκB and cAMP response element-binding (Creb) transcription factors, whereas MHC II gene expression is modulated by pathways initiated by interferon gamma (Ifnγ), TNF-α and TGF-β (reviewed by Ting & Baldwin, 1993). *E. coli* K1 colonization has a marked effect on the transcription of these important molecules; however, it is difficult to discern a meaningful pattern in the differential regulation observed in this study and their significance remains uncertain.

Transcriptomic data showed that *E. coli* K1 colonization resulted in the differential regulation of several factors involved in apoptotic pathways. Apoptosis is relevant to the intracellular phase of *E. coli* K1 infection as the pathogen has the capacity to prevent apoptotic initiation. This is achieved by the up-regulation of anti-apoptotic Bcl_{XL} which inhibits mitochondrial cytochrome *c* release (Sukumaran *et al.*, 2004). Therefore, it is interesting that P9 intestinal tissues up-regulated the expression of BH3 interacting-domain death agonist (Bid) and reticulon four (Rtn4). Bid promotes mitochondrial cytochrome *c* release (Zhao *et al.*, 2003) and Rtn4 inhibits the activity of Bcl_{XL} (Tagami *et al.*, 2000). In addition, several caspase genes and DNA fragmentation factor beta (Dffb), a nuclease which targets cellular DNA during apoptosis (Liu *et al.*, 1997), were also up-regulated. This pattern of pro-apoptotic gene regulation was absent from the transcriptome of neonates colonized at P2. No changes in Bcl_{XL} expression were detected in this group; however, a fourfold up-regulation of apoptosis inhibitor 3 (Iap3) was detected. Iap3 is a potent inhibitor of caspase-mediated apoptotic pathways (reviewed by Deveraux & Reed, 1999). The prevention of apoptosis is essential for the

intracellular survival of *E. coli* K1. This data indicates that neonates that are refractive to systemic disease can increase expression of factors which are antagonistic to the anti-apoptotic mechanism used by *E. coli* K1.

There was no evidence that *E. coli* K1 intestinal colonization affected expression of genes encoding Tff3, Fcgbp or any of the gel-forming intestinal mucins. However, the trefoil factor Tff2 was significantly down-regulated in the intestines of P2 neonates 24 h after colonization. In adults, Tff2 is primarily found in the stomach and is not conventionally associated with the lower GI tract (reviewed by Thim, 1997). The regulation of intestinal Tff3 expression in the foetus and neonate has been described previously (Lin *et al.*, 1999; Mashimo *et al.*, 1995) and Tff2 expression has been shown in foetal intestinal tissues (Samson *et al.*, 2011); however, the post-natal expression of this peptide has not been examined in the neonatal rat. The data presented here demonstrate that Tff2 is transiently up-regulated in the neonatal intestine and that this increased expression is maintained over the P3-P9 period; however, expression declined substantially after P9. It is interesting to note that the period of increased Tff2 expression exactly matches the period in which the neonatal rat develops resistance to systemic *E. coli* K1 infection. Whilst one cannot assume a causal relationship between these observations, the fact that *E. coli* K1 colonization abolishes the normal pattern of Tff2 expression demonstrates that a link does exist. The observation that IL-1β secretion and NFκB activation are increased immediately prior to the down-regulation of Tff2 provides a mechanistic basis for the down-regulation of Tff2 (Dossinger *et al.*, 2002).

Trefoil peptides have multiple functions, which include regulation of healing, inflammation and the immune response (Playford *et al.*, 1995; Tran *et al.*, 1999; Kurt-Jones *et al.*, 2007) as well as a structural role in the cytoprotective mucus barrier (Thim *et al.*, 2004; Kjellev *et al.*, 2006; Playford *et al.*, 2006; Yu *et al.*, 2011). Any of these functions could be relevant to *E. coli* K1 infection. The potential role of Tff2 in the intestines can be ascertained from data obtained from Tff2-KO animals (Kurt-Jones *et al.*, 2007). A potentially significant observation is that these animals are more susceptible to dextran sodium sulphate (DSS)-induced colitis than their wild-type counterparts. DSS is commonly used to induce experimental colitis but the mechanism of action has only recently been determined. Oral administration of DSS disrupts the stability of the inner stratified mucus layer, allowing luminal bacteria to access the colonic epithelium (Johansson *et al.*, 2010). One interpretation of these studies is that

Tff2 enhances the stability of the mucus layer. Therefore, the developmental increase in Tff2 observed in this investigation may form a component of colonic mucus barrier development in the neonate. Furthermore, if *E. coli* K1 colonization disrupts this developmental process it would compromise barrier development and allow the pathogen to access the colonic epithelium and, potentially, invade the host tissue. The reduction in detectable Muc2 protein 48 h after colonization of P2 neonates demonstrates that *E. coli* K1 modulates Muc2 and thus provides preliminary evidence supporting this hypothesis.

Although the data presented in this chapter do indicate several areas of potential interest with regards to *E. coli* K1 colonization of the intestinal tract there are several caveats which must be taken into account. Due to a limited number of GeneChip arrays the transcriptomic data was based on a single microarray per experimental group. RNA samples from multiple animals were pooled in order to provide data approximating the mean transcriptomic response of the tissues analysed; however, it is possible that much of the differential regulation indicated by this analysis would not be identified as statistically significant by a similar analysis using multiple replicate arrays. Another important issue is the fact that all the results described here are based on the analysis of whole intestinal tissues. The intestine is a large multi-compartmental structure with each compartment comprising distinct tissues. Therefore, treating these tissues as a single unit carries inherent risks with respect to the quantification of gene expression. For example the up-regulation of a gene in one compartment may be masked by the down-regulation of the same gene in a different compartment and *vice-versa*. Furthermore, the extraction of RNA and protein from whole intestinal tissues means that these samples would have primarily represented the ileal and jejunal tissues, as these are by far the largest structures within the intestine. This bias could have influenced the results of the assays described in this chapter. The intestine is also directly linked to the stomach and pancreas. Although great care was taken to avoid contamination of intestinal samples by these tissues, the risk of carry-over must be considered. Finally, it is necessary to note that whilst the data presented here demonstrate a correlative link between *E. coli* K1 colonization and the tissue responses observed they do not establish a causative link between those responses and the progression of *E. coli* K1 infection.

CHAPTER 5

GENERAL DISCUSSION

The data presented in this thesis represents an attempt to provide a mechanistic basis for the age-dependency of systemic *E. coli* K1 infection in the neonate. The results and potential limitations of the experiments described in the preceding chapters have been previously discussed. Therefore, this chapter will provide an overview of the data and the potential implications for our current understanding of neonatal *E. coli* K1 infection. Avenues for future investigations are also suggested and examples of ongoing research, based on the results of this investigation, are included.

The data presented in Chapter 2 provide compelling evidence that the intestine is the basis of age-dependency in the neonatal rat model, as indicated by previous investigators (Glode *et al.*, 1977; Mushtaq *et al.*, 2005). The intestinal tract is a highly complex environment and our current understanding of it is incomplete. Complexity is conferred by the tissues, comprising multiple cell types and interwoven with the enteric nervous system and GALT, and the trillions of microbes that comprise the microbiota. The intestinal tissues require this complexity in order to fulfil their function as an active interface between the host and the external environment. In turn, the microbiota is complex due to the multiple ecological niches provided by the enteric milieu. These factors make understanding the intestinal environment a challenge. The *post-partum* development of both tissue and microbiota which occurs in the neonate adds another layer of variability which also had to be taken into account during this investigation.

The previous decade has seen multiple studies which have used culture-independent methods to highlight developmental aspects of the intestinal microbiota (for example Favier *et al.*, 2002; Palmer *et al.*, 2007). This research has significantly broadened our understanding of the different taxonomic groups which inhabit the intestine and the temporal trends which affect the overall composition of the microbial population. The mCR function provided by the commensal microbiota is undoubtedly of great importance in protecting the host from colonization by obligate and opportunistic pathogens (Hooper *et al.*, 2003; Endt *et al.*, 2010; Vaishnava *et al.*, 2011). This protective role is complemented by the capacity of the microbiota to modulate the virulence of some opportunistic pathogens (Bernet *et al.*, 1993; Coconnier *et al.*, 2000; Altenhoefer *et al.*, 2004). These concepts formed the basis of the hypothesis that the microbiota is a key factor in modulating susceptibility to *E. coli* K1 infection.

Quantitative and qualitative analysis of the microbiota of both susceptible and refractive neonates failed to identify any immediately obvious differences that could account for the age dependency of systemic infection. However, the expansion of two clostridial genera over the P2-P9 period potentially represents a population shift of relevance to *E. coli* K1 colonization (Itoh & Freter, 1989). The significance of these bacteria could be determined by feeding susceptible neonates with *Clostridia* followed by an assessment of the impact on susceptibility to *E. coli* K1 infection. Such an approach has previously been used to demonstrate the protective effects of *Lactobacillus* spp. (Lee *et al.*, 2000). However, the feasibility of this method with respect to *Clostridia* is questionable as the oxygen tension of the P2 neonatal intestine may be higher than that of the P9 equivalent and would thus represent a less hospitable environment for obligate anaerobic bacteria.

Antibiotic suppression of the endogenous microbiota was used to assess the potential role of direct and competitive mCR mechanisms in modulating susceptibility to *E. coli* K1 infection. Suppression of the microbiota had no discernible impact on susceptibility to *E. coli* K1, providing further evidence that it does not play a direct role in modulating the capacity of the pathogen to cause systemic disease. As previously stated, the results of the suppression study must be treated with an element of caution as the antibiotic-resistant A192PPR transformant was notably less virulent than the parent A192PP isolate. A192PPR is capable of causing systemic disease in neonates colonized at P2; however, its capacity to cause systemic disease in the extraintestinal environment of refractive neonates was not assessed in the current study. Systemic administration of A192PPR to P9 neonates would serve as a useful validation of this method and the conclusions based on it.

Colonization by the microbiota stimulates a number of hCR mechanisms, including the secretion of inducible AMPs and production of sIgA (Hooper *et al.*, 2003; Macpherson & Uhr, 2004; Vaishnava *et al.*, 2011). This aspect of mCR was not assessed in the current study and may be of significance. To date, no investigations have compared the susceptibility of GF and conventionally reared animals to *E. coli* K1 infection. Such a comparison would clarify whether or not neonates require the stimulation of hCR mechanisms initiated by bacterial colonization in order to develop resistance to *E. coli* K1.

Overall, the data does not support the hypothesis that the development of the neonatal intestinal microbiota modulates susceptibility to *E. coli* K1 infection. However, the methods used in this investigation do not take into account any spatial aspects of the microbial population. The individual GI compartments have distinct biochemical and physical properties and thus represent different ecological niches. Accordingly, the gastric, small intestinal and colonic compartments each play host to a partially distinct subset of the GI microbiota (Eckburg *et al.*, 2005; Hayashi *et al.*, 2005; Bik *et al.*, 2006). It is possible that significant changes to the composition of the microbiota of these distinct regions occurs over the P2-P9 neonatal period and that this variation was not detected by the methods employed here. The *post-partum* development of the microbiota of the different GI compartments has not been well characterized and is worthy of further investigation.

The intestinal tissues are subject to significant *post-partum* developmental alterations in response to exposure to the extra-uterine environment and initiation of enteral feeding. This development includes the proliferation of two secretory epithelial cell lineages which play a key role in maintaining intestinal barrier function in the small intestinal and colonic compartments. The colonic goblet cell population continues to expand *post-partum* and this expansion is accompanied by increased production of Muc2 and trefoil peptides (Chambers *et al.*, 1994; Fanca-Berthon *et al.*, 2009). The small intestinal Paneth cell population also grows rapidly in the post-natal period (Bry *et al.*, 1994), as does their secretion of AMPs (Mallow *et al.*, 1996). The proteins secreted by these cells are vital for maintaining the microbiota at a safe distance from the enteric epithelial surface (Johansson *et al.*, 2008; Vaishnava *et al.*, 2011). The fact that they are developmentally regulated therefore indicates that the intestinal barrier function in younger neonates is immature. This concept partly informed the hypothesis that the development of the neonatal intestine over the P2-P9 period modulated susceptibility to *E. coli* K1 infection.

The neonatal intestinal tract grew substantially from P2-P9. This growth was accompanied by a significant degree of developmental gene regulation, including a sustained increase in AMP expression and a transient increase in the trefoil peptide Tff2. Based on these results, as well as our current state of knowledge regarding the developmental regulation of Paneth and goblet cells, we can formulate a speculative model of intestinal barrier development over the P2-P9 period (Figure 5.1).

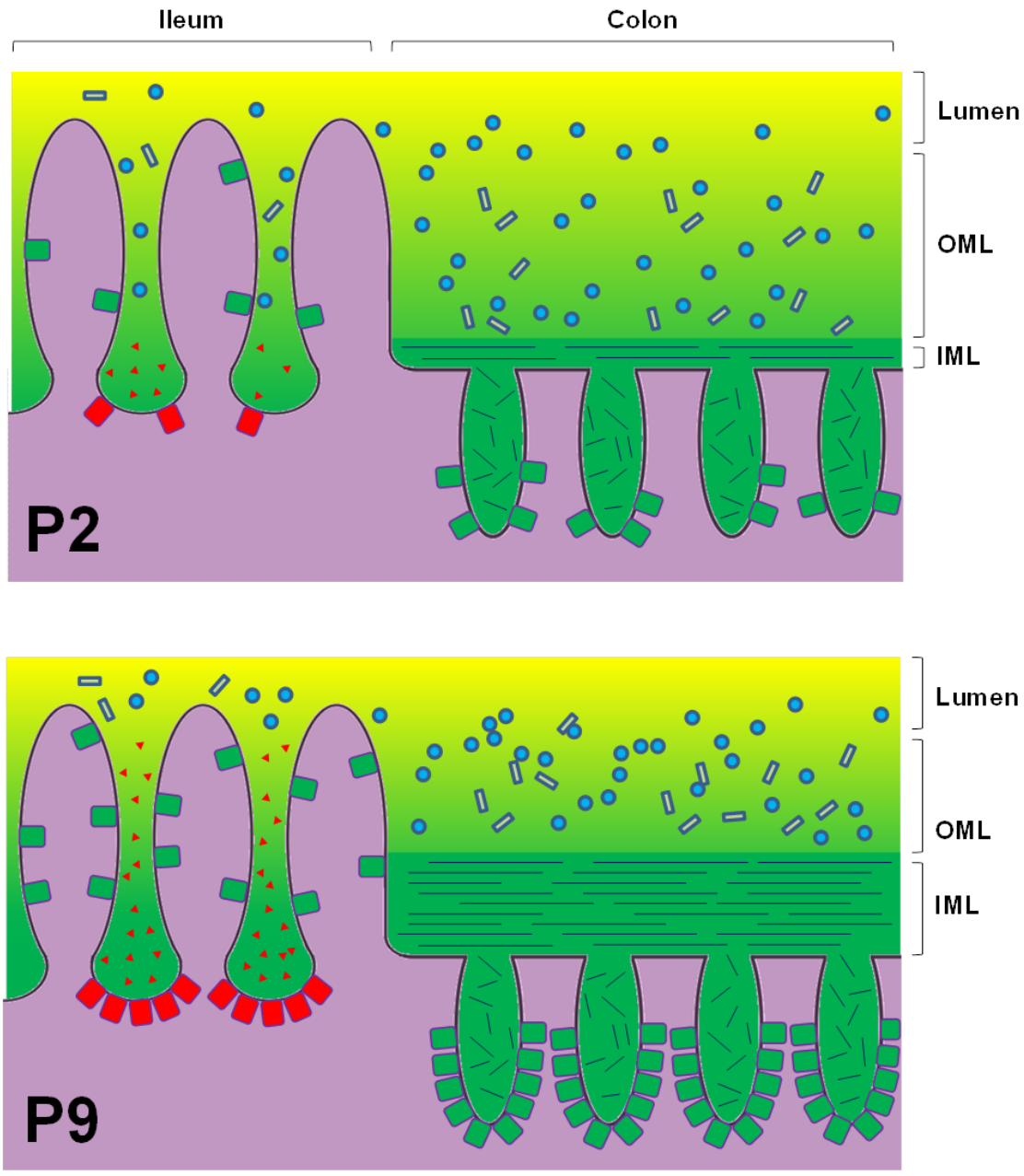


Figure 5.1: *Development of innate defence barriers in the neonatal intestine from P2-P9. The P2 ileum produces less defensin peptides than the more mature P9 tissues. The P2 colon produces less Muc2 and trefoil factor than the P9 colon, resulting in a less developed stratified inner mucus layer (IML) in the P2 compared to P9 colon. These deficiencies allow a closer association between the intestinal microbiota (which inhabit the outer mucus layer; OML) and the intestinal epithelium in P2 compared to P9 neonates.*

The comparative lack of defensin expression in P2 compared to P9 tissues is a strong indication that the AMP-dependent barrier function of the small intestine is weaker at P2 compared to P9. The development of the colonic mucus barrier may be related to the transient increase in Tff2 expression from P2-P9. It is interesting to note that expression of Tff3 in the rat colon does not start to increase towards adult levels until P12-P17 (Lin *et al.*, 1999; Fanca-Berthon *et al.*, 2009). Given the apparent role of Tff3 as a structural component of the colonic mucus barrier (Yu *et al.*, 2011), this pattern of developmental expression seems unusual. We can speculate that Tff2 plays a similar role to Tff3 in the early neonatal intestine. The peptide may stabilize the developing colonic mucus barrier prior to the developmental increase in Tff3 expression. However, tissue-specific aspects of developmental gene expression were not assessed in this investigation. Therefore, Tff2 may be localized to the small intestine rather than the colon. This issue could be easily resolved by analysis of mRNA and protein isolated from individual intestinal compartments by methods described in this investigation. Furthermore, the role of Tff2 in formation of the mucus barrier could be ascertained using a Tff2-KO animal model. The *post-partum* proliferation of goblet cells and developmental regulation of Muc2 and trefoil peptide expression strongly indicate that the colonic mucus barrier develops in the postnatal period. This implies that the barrier may be weaker in P2 compared to P9 neonates. The mucus barrier has previously been characterized using immunohistological methods (Johansson *et al.*, 2008) and these would also allow qualitative comparison of the P2 and P9 colonic mucus barrier.

The transcriptional responses of P2 and P9 intestinal tissues to colonization by *E. coli* K1 were highly divergent. Several genes encoding products involved in host defence mechanisms were differentially expressed, including developmentally regulated defensins and Tff2. The fact that comparatively few differentially expressed genes were shared between colonized P2 and P9 neonates indicates that the intestinal tissue of the refractive neonate responds very differently to that of the susceptible neonate. This demonstrates that the capacity of the host to respond to *E. coli* K1 colonization is likely to be a key factor in determining susceptibility to systemic infection. The suppression of Tff2 and loss of Muc2 protein in P2-colonized tissues may allow *E. coli* K1 access to the intestinal epithelium. Conversely, the up-regulation of defensin peptides by P9-colonized tissues may inhibit this interaction (Figure 5.2).

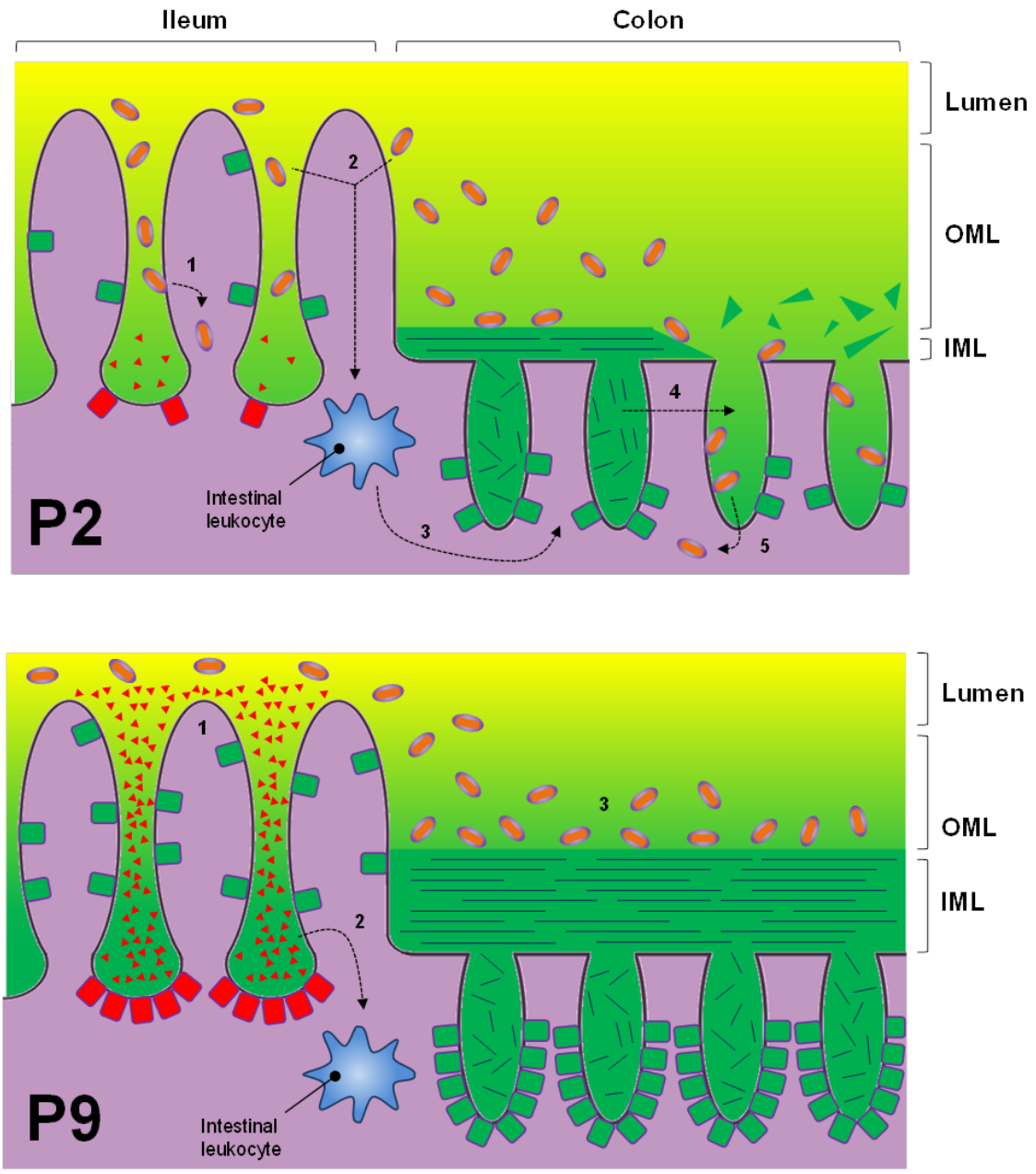


Figure 5.2: *Colonization of the P2 and P9 intestine by E. coli K1. (P2) 1; defensin deficiency allows bacteria to access /invade the small intestinal tissues. 2; bacterial colonization is detected by intestinal leukocytes. 3; activated leukocytes secrete IL-1β which activates NFκB transcription factor. 4; activated NFκB suppresses trefoil factor production in goblet cells, resulting in breakdown of the inner mucus layer (IML) structure. 5; Loss of IML integrity allows bacteria to access/invade colonic tissue. (P9) 1; up-regulated defensin production prevents bacteria accessing the small intestinal tissues. 2; defensins prevent IL-1β secretion by leukocytes. 3; IML prevents bacteria accessing the colonic tissue.*

E. coli K1 colonization of the P2 intestine invariably results in translocation of the pathogen from the intestine into the systemic circulation. However, a key question has yet to be resolved: where in the intestine does translocation occur? This specific issue was not addressed by this investigation; however, the developmental deficiencies that are likely to be present in both the small intestinal and colonic barrier function of the P2 intestine indicate that both of these regions represent a potential route of invasion. The lack of secreted defensins could allow *E. coli* K1 to access the small intestinal epithelium. Equally, dysregulation of Tff2 expression may provide access to the colonic epithelium. Colonization of the P2 intestine induces the secretion of IL-1β. This is most likely due to the detection of PAMPs (for example LPS) by the intestinal leukocyte population. Adult intestinal macrophages lack the CD14 receptor which systemic macrophages use to detect bacterial LPS (Smythies *et al.*, 2005). LPS tolerance prevents intestinal macrophages from inducing potentially damaging inflammatory reactions in response to the intestinal microbiota. This tolerance does not develop until the peri-natal period (Lotz *et al.*, 2006; Maheshwari *et al.*, 2011) and may explain why IL-1β secretion is not induced in P9 neonates. Furthermore, α-defensins represent another potential inhibitor of IL-1β secretion from macrophages in the P9 intestine (Shi *et al.*, 2007). Secretion of IL-1β in P2 intestines colonized by *E. coli* K1 results in activation of NFκB and transcriptional suppression at the Tff2 promoter. The decrease in detectable Muc2 protein, subsequent to the suppression of Tff2, may indicate that loss of the trefoil peptide results in a breakdown of the colonic mucus barrier. This would allow access to the colonic epithelium and, potentially, result in *E. coli* K1 invasion *via* this route.

The site of *E. coli* K1 translocation is an important unknown in the pathogenesis of this organism. Thorough histological analysis of colonized intestinal tissues would be an ideal method of resolving this issue. The importance of α-defensin production could also be assessed experimentally. This can be achieved by selective ablation of Paneth cells using the zinc-binding dye dithizone (Sherman et al, 2005). The proposed mechanism of colonic invasion could also be examined with relative ease. The effects of *E. coli* K1 colonization on the stability of the colonic mucus barrier could be assessed using the immunohistological methods described previously (Johansson *et al.*, 2008) and Tff2 protein could be simultaneously localized. Flow cytometry could be used to compare the number of $CD14^+$ macrophages present in P2 and P9 intestinal tissues to

determine if the P2 intestine is more susceptible to LPS-induced inflammation that the tissues of older animals. Furthermore, the isolation of intestinal macrophage populations by fluorescence-activated cell sorting (FACS) could be used to determine if *E. coli* K1 uses this large reservoir of leukocytes for systemic growth prior to haematogenous dissemination in susceptible neonates.

Some preliminary progress has already been made regarding these avenues of investigation. Colonization of the small intestine by *E. coli* K1 is currently under investigation in our laboratory at the UCL School of Pharmacy. Preliminary data indicate that there are differences in the capacity of *E. coli* K1 to colonize the non-colonic GI compartments of P2 and P9 neonates (Figure 5.3). The higher *E. coli* K1 load detected in the proximal (and to a lesser extent distal) small intestine of P2 neonates compared to P9 neonates may indicate that the small intestine is a more likely site of bacterial translocation. Furthermore, dithizone treatment has been successfully used to significantly reduce Defa24 and Defa-rs1 expression in P9 neonates. This will provide a useful model in which to determine the importance of these peptides in modulating susceptibility to *E. coli* K1.

The neonatal colonic mucus barrier is currently under investigation in collaboration with the Mucin Biology Group at the University of Gothenburg. Preliminary data shows that the stratified inner mucus layer, which confers the colonic barrier function, is almost entirely absent in the P2 colon but is present in the P9 colon (Figure 5.4). This supports the developmental model illustrated in Figure 5.1. Intriguingly, colonization of P2 animals with *E. coli* K1 appears to result in a massive decrease in Muc2 stored in colonic goblet cells. This effect is not evident in neonates colonized at P9. This observation could mean that *E. coli* K1 colonization either suppresses Muc2 synthesis or induces goblet cells to dump their stored Muc2 into the intestinal lumen. The latter may represent an attempt by the host to clear the pathogen from the intestines, an effect which has been observed during colonization by the rodent intestinal pathogen *Citrobacter rodentium* (Linden *et al.*, 2008; Bergstrom *et al.*, 2010). However, the loss of stored Muc2 at such an early stage in the development of the colonic mucus barrier would be likely to compromise this developmental process. These results indicate that the colon represents a possible route of infection for *E. coli* K1.

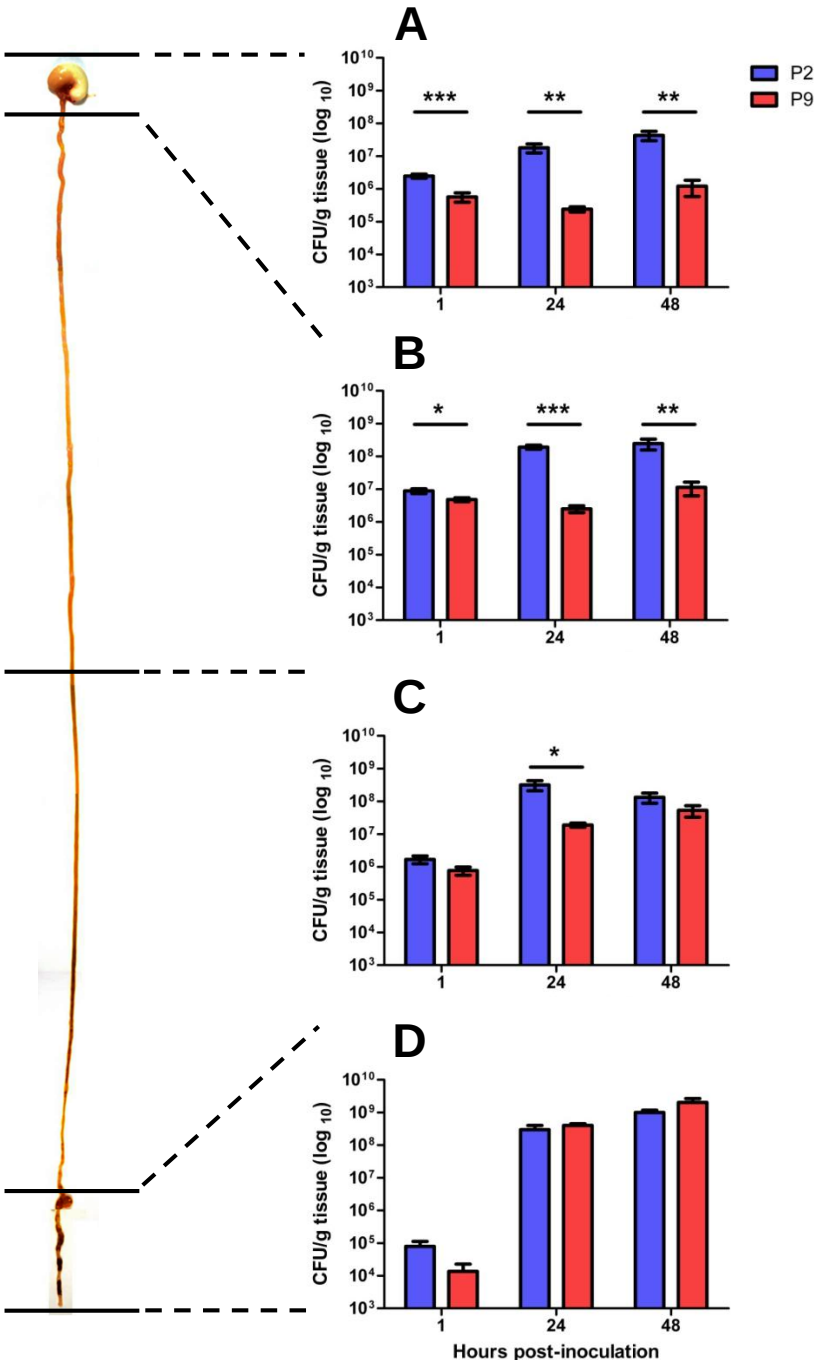


Figure 5.3: *Quantification of E. coli K1 from the GI compartments of P2 and P9 neonates. Statistically significant differences are indicated; Mann-Whitney (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Data provided by Fatma Dalgakiran (UCL School of Pharmacy).*

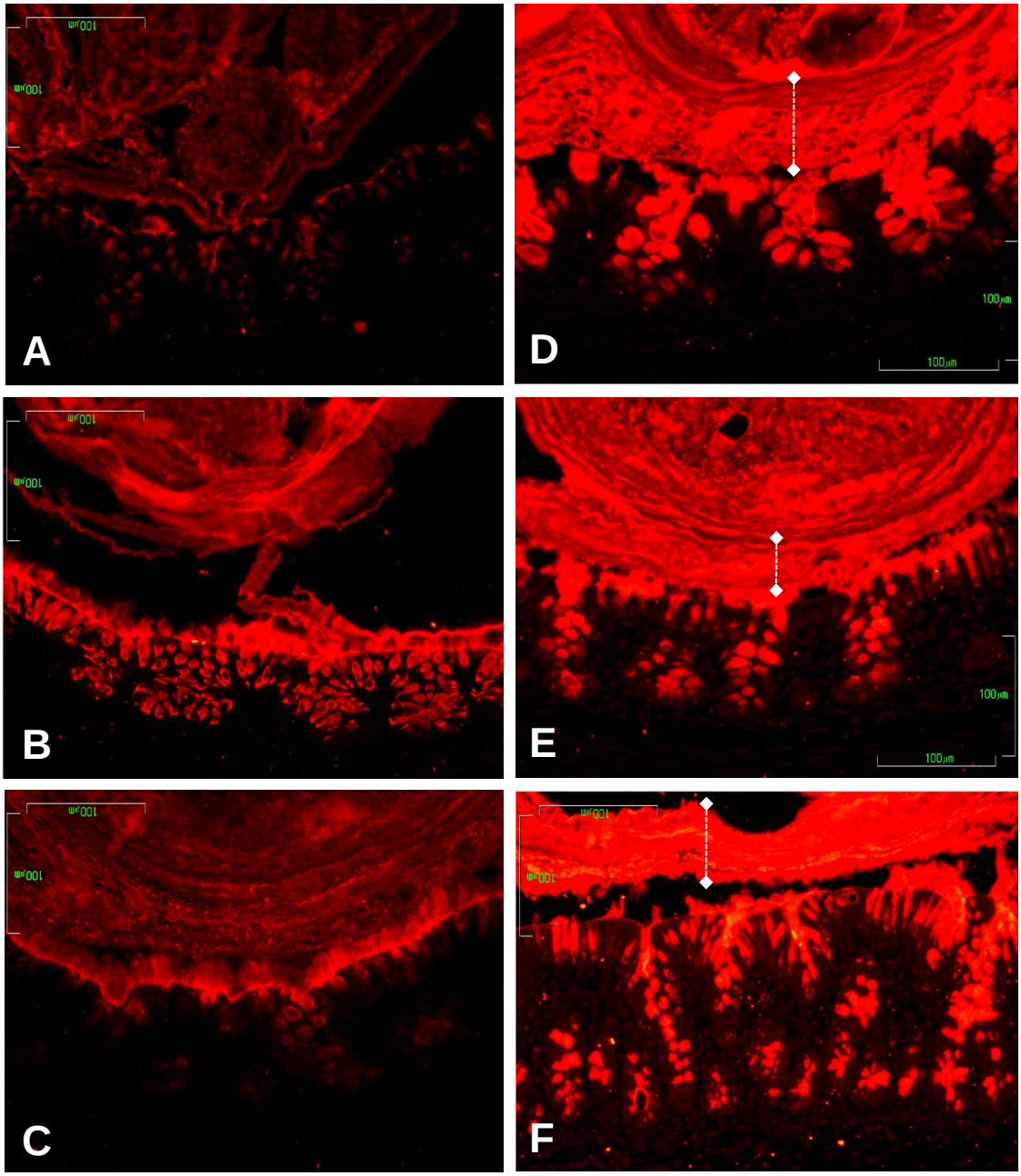


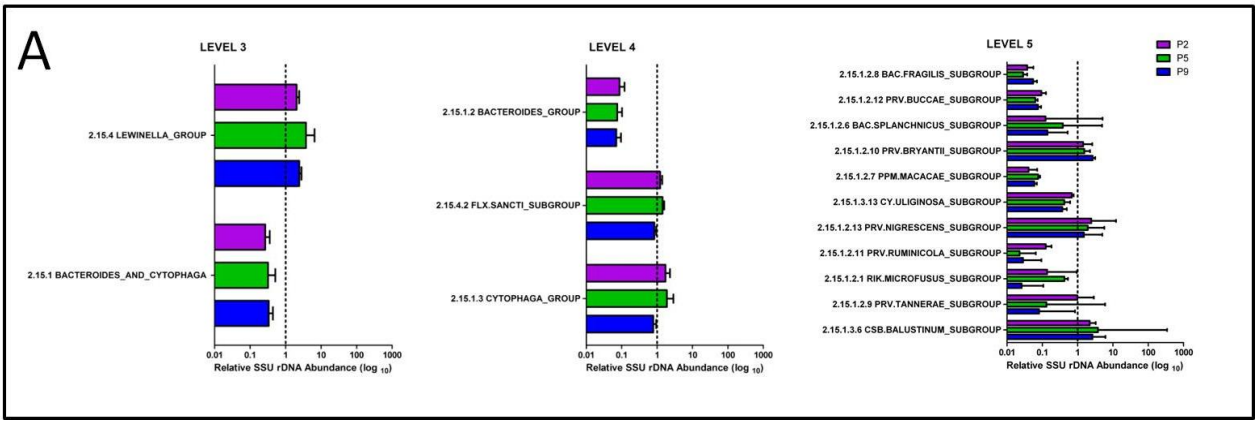
Figure 5.4: *The Muc2 colonic mucus barrier in P2 and P9 neonates. Methacarn-fixed colonic tissues from P2 (A) and P9 (D) neonates were stained for Muc2. Colonic tissues were obtained from P2 (B/C) and P9 (E/F) neonates 48 h after inoculation with E. coli K1 (C/F) or sterile broth (B/E). All images were processed in the same way. The stratified inner mucus layer is indicated in D, E and F (----). Scale bars represent 100 µm. Images supplied by Malin Johansson (University of Gothenburg).*

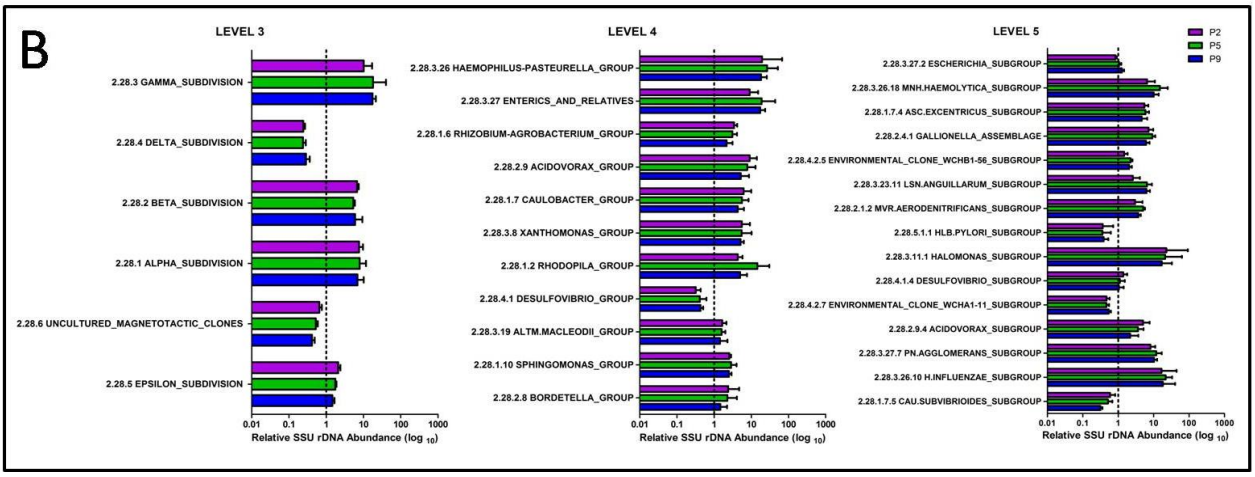
The aim of this investigation was to determine the influence of the developing intestinal microbiota and maturing intestinal tissues on the capacity of *E. coli* K1 to translocate from the neonatal intestine into the systemic circulation. The results presented in this thesis strongly support the hypothesis that maturation of the innate defensive mechanisms of the neonatal intestine accounts for the development of resistance to systemic *E. coli* K1 infection. However, they do not preclude a role for the microbiota in the stimulation of this developmental process. Although the mechanics of susceptibility and resistance to *E. coli* K1 infection have not been conclusively identified by this investigation, it has provided some interesting avenues of future research. In addition, depending on the outcome of that research, both Tff2 and α-defensin AMPs represent potential therapeutic candidates for the prevention of sepsis and NBM mediated by *E. coli* K1.

AMPs are now recognized as a potential replacement for standard antibiotics (reviewed by Hancock & Sahl, 2006). Recombinant α-defensin-like peptides could be used to supplement the neonatal GI tract with AMPs and boost the barrier function of the small intestine. However, to date, AMPs have only been successfully used in topical applications and have suffered from production problems, toxicity and unfavourable pharmacokinetics. Conversely, recombinant Tff2 is easily produced in bacterial expression vectors (Sun *et al.*, 2010), is highly stable in the GI tract (Kjellev *et al.*, 2007) and has been successfully used to treat experimental GI injuries (Poulsen *et al.*, 1999; Tran *et al.*, 1999; Sun *et al.*, 2009). The dysregulation of Tff2 expression by *E. coli* K1 colonization of the neonatal intestine could be compensated by oral administration of the recombinant protein. If Tff2 does play a significant role in stabilizing the colonic mucus barrier, or modulates infection by an alternate mechanism, this could provide a novel therapeutic strategy for combating neonatal mortality.

APPENDICES

Appendix A


A
LEVEL 3
2.15.4 LEWINELLA_GROUP
2.15.1 BACTEROIDES_AND_CYTOPHAGA
LEVEL 4
2.15.1.2 BACTEROIDES_GROUP
2.15.4.2 FLX.SANCTI_SUBGROUP
2.15.1.3 CYTOPHAGA_GROUP
LEVEL 5
P2
P5
P9
2.15.1.2.8 BAC.FRAGILIS_SUBGROUP
2.15.1.2.12 PRV.BUCCAE_SUBGROUP
2.15.1.2.6 BAC.SPLANCHNICUS_SUBGROUP
2.15.1.2.10 PRV.BRYANTII_SUBGROUP
2.15.1.2.7 PPM.MACACAE_SUBGROUP
2.15.1.3.13 CY.ULIGINOSA_SUBGROUP
2.15.1.2.13 PRV.NIGRESCENS_SUBGROUP
2.15.1.2.11 PRV.RUMINICOLA_SUBGROUP
2.15.1.2.1 RIK.MICROFUSUS_SUBGROUP
2.15.1.2.9 PRV.TANNERAE_SUBGROUP
2.15.1.3.6 CSB.BALUSTINUM_SUBGROUP
0.01 0.1 1 10 100 1000
Relative SSU rDNA Abundance (log 10)


B
LEVEL 3
2.28.3 GAMMA_SUBDIVISION
2.28.4 DELTA_SUBDIVISION
2.28.2 BETA_SUBDIVISION
2.28.1 ALPHA_SUBDIVISION
2.28.6 UNCULTURED_MAGNETOTACTIC_CLONES
2.28.5 EPSILON_SUBDIVISION
LEVEL 4
2.28.3.26 HAEMOPHILUS-PASTEURELLA_GROUP
2.28.3.27 ENTERICS_AND_RELATIVES
2.28.1.6 RHIZOBIUM-AGROBACTERIUM_GROUP
2.28.2.9 ACIDOVORAX_GROUP
2.28.1.7 CAULOBACTER_GROUP
2.28.3.8 XANTHOMONAS_GROUP
2.28.1.2 RHODOPILA_GROUP
2.28.4.1 DESULFOVIBRIO_GROUP
2.28.3.19 ALTM.MACLEODII_GROUP
2.28.1.10 SPHINGOMONAS_GROUP
2.28.2.8 BORDETELLA_GROUP
LEVEL 5
P2
P5
P9
2.28.3.27.2 ESCHERICHIA_SUBGROUP
2.28.3.26.18 MNH.HAEMOLYTICA_SUBGROUP
2.28.1.7.4 ASC.EXCENTRICUS_SUBGROUP
2.28.2.4.1 GALLIONELLA_ASSEMBLAGE
2.28.4.2.5 ENVIRONMENTAL_CLONE_WCHB1-56_SUBGROUP
2.28.3.23.11 LSN.ANGUILLARUM_SUBGROUP
2.28.2.1.2 MVR.AERODENITRIFICANS_SUBGROUP
2.28.5.1.1 HLB.PYLORI_SUBGROUP
2.28.3.11.1 HALOMONAS_SUBGROUP
2.28.4.1.4 DESULFOVIBRIO_SUBGROUP
2.28.4.2.7 ENVIRONMENTAL_CLONE_WCHA1-11_SUBGROUP
2.28.2.9.4 ACIDOVORAX_SUBGROUP
2.28.3.27.7 PN.AGGLOMERANS_SUBGROUP
2.28.3.26.10 H.INFLUENZAE_SUBGROUP
2.28.1.7.5 CAU.SUBVIBRIOIDES_SUBGROUP
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Relative SSU rDNA Abundance (log 10)

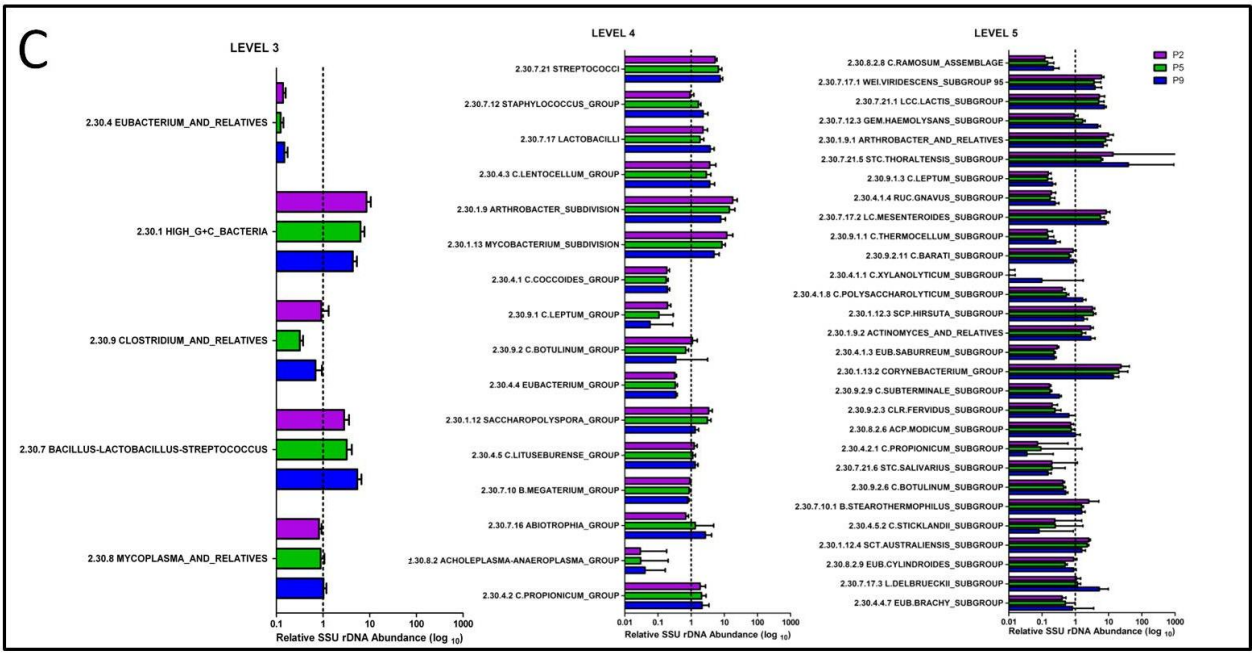
C
LEVEL 3
2.30.4 EUBACTERIUM_AND_RELATIVES
2.30.1 HIGH_G+C_BACTERIA
2.30.9 CLOSTRIDIUM_AND_RELATIVES
2.30.7 BACILLUS-LACTOBACILLUS-STREPTOCOCCUS
2.30.8 MYCOPLASMA_AND_RELATIVES
LEVEL 4
2.30.7.21 STREPTOCOCCI
2.30.7.12 STAPHYLOCOCCUS_GROUP
2.30.7.17 LACTOBACILLI
2.30.4.3 C.LENTOCELLUM_GROUP
2.30.1.9 ARTHROBACTER_SUBDIVISION
2.30.1.13 MYCOBACTERIUM_SUBDIVISION
2.30.4.1 C.COCCOIDES_GROUP
2.30.9.1 C.LEPTUM_GROUP
2.30.9.2 C.BOTULINUM_GROUP
2.30.4.4 EUBACTERIUM_GROUP
2.30.1.12 SACCHAROPOLYSPORA_GROUP
2.30.4.5 C.LITUSEBURENSE_GROUP
2.30.7.10 B.MEGATERIUM_GROUP
2.30.7.16 ABIOTROPHIA_GROUP
2.30.8.2 ACHOLEPLASMA-ANAEROPLASMA_GROUP
2.30.4.2 C.PROPIONICUM_GROUP
LEVEL 5
P2
P5
P9
2.30.8.2.8 C.RAMOSUM_ASSEMBLAGE
2.30.7.17.1 WEI.VIRIDESCENS_SUBGROUP 95
2.30.7.21.1 LCC.LACTIS_SUBGROUP
2.30.7.12.3 GEM.HAEMOLYSANS_SUBGROUP
2.30.1.9.1 ARTHROBACTER_AND_RELATIVES
2.30.7.21.5 STC.THORALTENSIS_SUBGROUP
2.30.9.1.3 C.LEPTUM_SUBGROUP
2.30.4.1.4 RUC.GNAVUS_SUBGROUP
2.30.7.17.2 LC.MESENTEROIDES_SUBGROUP
2.30.9.1.1 C.THERMOCELLUM_SUBGROUP
2.30.9.2.11 C.BARATI_SUBGROUP
2.30.4.1.1 C.XYLANOLYTICUM_SUBGROUP
2.30.4.1.8 C.POLYSACCHAROLYTICUM_SUBGROUP
2.30.1.12.3 SCP.HIRSUTA_SUBGROUP
2.30.1.9.2 ACTINOMYCES_AND_RELATIVES
2.30.4.1.3 EUB.SABURREUM_SUBGROUP
2.30.1.13.2 CORYNEBACTERIUM_GROUP
2.30.9.2.9 C.SUBTERMINALE_SUBGROUP
2.30.9.2.3 CLR.FERVIDUS_SUBGROUP
2.30.8.2.6 ACP.MODICUM_SUBGROUP
2.30.4.2.1 C.PROPIONICUM_SUBGROUP
2.30.7.21.6 STC.SALIVARIUS_SUBGROUP
2.30.9.2.6 C.BOTULINUM_SUBGROUP
2.30.7.10.1 B.STEAROTHERMOPHILUS_SUBGROUP
2.30.4.5.2 C.STICKLANDII_SUBGROUP
2.30.1.12.4 SCT.AUSTRALIENSIS_SUBGROUP
2.30.8.2.9 EUB.CYLINDROIDES_SUBGROUP
2.30.7.17.3 L.DELBRUECKII_SUBGROUP
2.30.4.4.7 EUB.BRACHY_SUBGROUP
0.01 0.1 1 10 100 1000
Relative SSU rDNA Abundance (log 10)

Figure A1: *Comparative sub-phylum phylogenetic analysis of the composition of the GI tract microbiota of P2, P5 and P9 neonates.* *Relative abundance of amplified SSU rDNA sequences from P2, P5 and P9 samples binding to class (level 3), order/family (level 4) and genus (level 5) taxonomic level microarray probes from the Bacteroidetes (A), Proteobacteria (B) and Gram-positive (C) phyla. P2, P5 and P9 neonatal data were normalized to adult data as indicated by the dashed lines at x=1. Numeric codes correspond to prokMSA (http://greengenes.lbl.gov) SSU rDNA database classifications for the indicated taxonomic level probes. Probes were ranked according to average Cy5 and Cy3 fluorescence across the P2, P5, and P9 datasets, with the highest at the top of each figure. Error bars are SEM from four arrays.*

Table A1: *Comparative species level analysis of the GI tract microbiota of P2, P5 and P9 neonates. Mean relative SSU rDNA abundance (RSA), RSA standard deviation from four arrays (Stdev) and statistical comparison to adult data determined by two-tailed t-test (p-value) are detailed.*

Name	P2			P5			P9		
	RSA	**StdDev**	***p*-value**	**RSA**	**StdDev**	***p*-value**	**RSA**	**StdDev**	***p*-value**
Euryarchaeote DJ3	8.63	4.90	1.81E-03	7.87	3.47	4.51E-04	5.82	2.31	7.77E-04
Verrucomicrobium DEV179	0.19	0.06	2.76E-04	0.14	0.10	7.61E-03	0.16	0.06	3.30E-03
Holophaga spp.	0.09	0.03	7.78E-05	0.09	0.03	8.55E-05	0.10	0.04	3.49E-04
AB113725 clone: OAB38	0.17	0.09	6.50E-04	0.10	0.02	1.62E-05	0.07	0.03	4.72E-05
Bacteroides merdae	0.02	0.03	2.46E-03	0.02	0.00	5.90E-13	0.02	0.03	8.56E-05
Bacteroides acidofaciens	0.03	0.06	1.21E-02	0.04	0.07	1.26E-02	0.03	0.09	1.35E-02
Bacteroides fragilis	0.04	0.10	2.10E-02	0.03	0.05	1.06E-02	0.05	0.21	3.89E-02
Bacteroides caccae	0.02	0.05	8.56E-03	0.02	0.01	1.62E-04	0.04	0.10	1.52E-02
Bacteroides vulgatus	0.02	0.04	6.24E-03	0.02	0.03	2.58E-03	0.03	0.09	2.21E-03
Bacteroides thetaiotaomicron	0.51	0.18	3.02E-03	0.65	0.27	5.92E-02	0.30	0.30	1.54E-02
Microcystis holsatica	1.33	0.18	4.86E-03	1.43	0.23	5.45E-03	1.16	0.73	6.37E-01
Microcystis elabens	1.68	0.34	1.04E-04	1.61	0.18	2.06E-07	1.18	0.48	4.34E-01
Leptospira santarosai	0.10	0.06	3.26E-03	0.09	0.03	4.36E-04	0.09	0.05	3.34E-03
Sphingomonas spp.	0.48	0.07	1.79E-06	0.56	0.16	3.91E-03	0.57	0.16	3.30E-03
Sphingomonas sp.	1.20	1.75	7.53E-01	3.54	0.97	1.65E-04	1.77	0.96	6.24E-02
Sinorhizobium meliloti	4.72	2.39	1.65E-03	3.76	2.44	8.39E-03	2.43	1.18	4.34E-03
Sinorhizobium fredii	0.67	0.16	3.37E-02	0.58	0.24	6.84E-02	0.31	0.07	5.23E-04
Rhizobium tropici	5.95	1.04	3.34E-05	4.33	0.95	1.76E-04	1.67	0.62	2.78E-02
Rhizobium mongolense	0.38	0.16	1.00E-02	0.59	0.43	1.97E-01	0.70	0.31	1.33E-01
Blastochloris sulfoviridis	0.51	0.37	1.31E-01	0.43	0.22	3.07E-02	0.33	0.11	2.58E-05
Paracoccus sp.	3.04	1.21	1.54E-04	1.98	1.21	4.75E-02	3.09	0.96	7.54E-05
L35465 clone SAR 122	0.98	0.40	9.03E-01	1.30	0.60	2.71E-01	1.33	0.37	7.03E-02
Microvirgula aerodenitrificans	3.29	7.04	1.62E-01	6.94	2.26	2.49E-04	4.33	1.73	1.11E-04
Burkholderia sp.	2.90	1.21	1.29E-02	2.79	0.56	1.88E-03	2.25	0.91	2.52E-02
AJ408960 clone HuCA4	0.07	0.06	1.78E-03	0.07	0.08	3.66E-03	0.05	0.05	4.67E-03
Zoogloea sp.	0.47	0.23	4.56E-02	0.32	0.13	1.04E-02	0.65	0.33	1.73E-01
Z93978 clone T35	0.62	0.21	6.28E-02	0.50	0.18	2.96E-02	0.54	0.21	4.77E-02
Pseudomonas fluorescens	2.21	0.45	4.36E-03	2.19	0.12	8.75E-05	2.20	0.76	1.82E-02
Pseudomonas sp.	1.64	0.91	1.26E-01	1.63	1.22	2.23E-01	1.26	0.97	5.36E-01
Alteromonas macleodii	0.85	0.63	6.63E-01	1.47	1.17	3.49E-01	1.80	0.49	1.14E-02
Shewanella frigidimarina	4.92	12.46	1.10E-01	16.21	52.87	3.77E-02	7.99	5.93	7.29E-03
Vibrio aerogenes	1.64	0.70	5.43E-02	1.88	1.48	1.56E-01	1.02	0.79	9.64E-01
Aeromonas jandaei	0.22	0.13	5.12E-03	0.35	0.12	8.13E-05	0.42	0.13	2.77E-04
Pasteurella sp.	5.89	10.41	4.73E-02	13.82	14.67	3.96E-03	8.90	15.44	1.92E-02
Pasteurella sp.	3.99	41.47	2.89E-01	22.93	40.86	1.32E-02	18.75	7.29	8.75E-04
Yersinia aldovae	0.93	0.37	7.13E-01	0.84	0.17	1.24E-01	0.79	0.25	1.68E-01

Yersinia frederiksenii	0.56	0.25	6.36E-02	0.91	0.34	6.31E-01	0.72	0.35	2.17E-01
Escherichia coli str.	4.01	15.62	1.94E-01	10.32	15.60	1.86E-02	10.12	14.52	1.90E-02
Escherichia coli str.	4.64	13.61	1.27E-01	9.69	36.32	6.73E-02	7.11	13.55	3.62E-02
Rhodobacter capsulatus	11.02	42.31	6.09E-02	27.75	85.44	2.09E-02	33.91	22.97	8.82E-06
Pantoea agglomerans	1.45	0.91	2.64E-01	2.05	1.25	6.33E-02	1.94	1.22	8.29E-02
Hydrocarbophaga effusa	7.11	18.18	1.39E-01	11.22	32.19	1.07E-01	25.97	9.70	4.08E-03
Stenotrophomonas sp.	16.37	16.83	3.34E-03	14.59	15.87	4.60E-03	4.45	4.99	3.26E-02
Desulfovibrio sp.	0.32	0.17	1.68E-02	0.70	0.79	4.88E-01	3.18	2.66	3.97E-02
Desulfovibrio sp.	0.07	0.05	3.12E-03	0.06	0.04	3.13E-03	0.12	0.17	2.13E-02
Desulfovibrio sp.	0.10	0.02	9.46E-09	0.11	0.02	3.03E-07	0.12	0.08	1.17E-04
Desulfovibrio sp.	0.24	0.13	9.93E-03	0.40	0.35	9.15E-02	0.33	0.18	1.12E-02
Cytophagales QSSC8L-9	0.19	0.09	3.78E-04	0.24	0.14	2.61E-03	0.28	0.16	1.52E-02
Helicobacter hepaticus str.	0.19	0.16	4.84E-03	0.33	0.26	1.14E-02	0.32	0.15	1.01E-03
Helicobacter hepaticus str.	1.16	1.14	7.24E-01	2.42	1.60	1.85E-02	2.23	1.13	1.01E-02
Helicobacter hepaticus str.	0.98	0.35	9.06E-01	1.22	0.44	2.84E-01	1.28	0.32	7.41E-02
Helicobacter pylori	3.01	0.35	4.46E-05	2.37	0.81	2.80E-03	1.20	0.24	1.36E-01
Clostridium rectum	4.42	1.99	2.73E-04	4.63	1.68	2.84E-03	1.59	1.22	2.59E-01
Propionibacterium cyclohexanicum	1.42	6.98	7.21E-01	0.61	0.21	1.48E-02	0.60	0.21	2.51E-02
Kibdelosporangium aridum	4.05	1.75	2.95E-03	4.06	2.77	1.47E-02	2.67	1.06	9.19E-04
Actinopolyspora mortivallis	0.20	0.05	5.59E-09	0.19	0.07	1.70E-05	0.25	0.17	8.30E-03
Kutzneria viridogrisea	0.21	0.08	6.64E-06	0.17	0.07	1.75E-06	0.23	0.27	3.67E-02
Mycobacterium tuberculosis	0.62	0.19	2.91E-02	0.76	0.14	1.40E-02	1.30	0.18	5.56E-03
Mycobacterium fortuitum	1.38	0.60	1.55E-01	1.56	0.92	1.40E-01	1.16	0.49	4.96E-01
Corynebacterium matruchotii	3.66	1.03	8.14E-03	15.39	70.15	2.53E-01	1.36	0.66	4.43E-01
Turicella sp.	4.62	2.52	2.36E-03	3.96	1.66	3.02E-04	1.64	0.72	4.20E-02
Corynebacterium flavescens	2.35	1.35	4.95E-02	2.20	1.19	5.29E-02	1.50	1.44	4.75E-01
Rhodococcus sp.	4.84	3.22	8.14E-04	4.10	2.67	1.45E-03	4.48	1.76	4.82E-05
Rhodococcus sp.	11.94	12.45	6.78E-04	11.33	9.93	5.42E-04	4.78	7.39	3.52E-02
Eggerthella lenta	0.09	0.04	1.41E-03	0.07	0.05	4.00E-03	0.08	0.03	6.16E-04
Geodermatophilus obscurus	10.02	11.85	1.45E-02	8.54	2.56	3.65E-06	5.25	5.81	2.58E-02
Streptomyces albus	1.67	0.66	7.43E-02	1.15	0.75	6.63E-01	0.88	0.43	6.20E-01
Streptomyces brasiliensis	3.99	3.15	1.85E-02	3.65	2.78	2.14E-02	4.08	2.69	1.79E-03
Streptomyces sp.	4.43	1.17	4.52E-07	4.20	1.48	5.35E-05	2.64	0.89	1.41E-03
Streptomyces salmonis	1.96	1.02	5.08E-02	1.44	0.59	1.33E-01	1.46	0.59	1.51E-01
Streptomyces sp.	0.72	12.65	8.02E-01	1.70	20.58	6.66E-01	1.71	8.95	5.98E-01
Streptosporangia spp.	1.56	0.61	5.04E-02	1.51	0.59	6.59E-02	1.00	0.96	9.98E-01
AF142943 PENDANT-31	1.50	0.55	1.00E-01	1.28	0.25	7.29E-02	1.80	1.35	1.30E-01
Arthrobacter QSSC8-13	9.43	1.11	3.88E-05	9.69	1.67	1.18E-04	3.87	1.47	5.15E-03
Arthrobacter sp.	2.94	1.01	3.62E-04	2.49	1.77	5.21E-02	1.22	0.61	4.27E-01
Arthrobacter haridrum	4.51	1.75	1.07E-05	4.23	2.02	2.29E-04	1.27	0.93	4.97E-01
Micrococcus QSSC8-1	0.35	0.16	8.52E-03	0.27	0.09	2.61E-05	0.46	0.16	2.30E-03
Arthrobacter sp.	0.74	0.34	2.14E-01	0.65	0.41	1.93E-01	1.08	0.53	7.64E-01
Kocuria varians	12.58	50.84	5.76E-02	9.45	6.79	5.82E-03	4.94	4.92	2.84E-02

Rothia amarae	0.52	0.26	6.58E-02	0.30	0.11	4.21E-03	0.65	0.27	6.98E-02
Actinomyces howellii	23.28	20.38	1.52E-03	22.11	18.36	1.29E-03	13.54	12.07	1.87E-03
Actinomyces sp.	4.99	2.34	1.24E-03	4.74	2.82	5.07E-03	3.30	2.07	1.25E-02
Bifidobacterium sp.	140.37	383.35	6.22E-03	152.89	353.87	4.54E-03	96.21	208.48	4.52E-03
Bifidobacterium magnum	0.70	0.48	2.85E-01	0.71	0.59	3.78E-01	0.62	0.44	1.72E-01
Kineosporia rhizophila	0.65	0.15	4.52E-03	0.64	0.19	2.03E-02	0.59	0.19	9.98E-03
Desulfotomaculum thermobenzoicum	7.08	5.27	3.92E-03	6.00	1.59	7.35E-07	4.47	1.96	9.79E-05
AF125206 clone I025	0.91	1.32	8.68E-01	0.72	4.30	7.51E-01	0.84	0.49	5.86E-01
AF068809 VC2.1	3.06	2.26	3.34E-02	2.30	2.18	1.18E-01	1.95	0.87	2.89E-02
AY192277 candidate division	19.08	23.73	8.85E-03	14.91	14.33	6.84E-03	9.74	18.93	3.57E-02
Phascolarctobacterium faecium	0.12	0.29	5.44E-02	0.10	0.26	4.59E-02	0.14	0.73	1.18E-01
Butyrivibrio fibrisolvens str.	0.09	0.02	1.17E-09	0.10	0.05	1.13E-03	0.08	0.20	5.70E-03
Butyrivibrio fibrisolvens str.	0.05	0.02	4.47E-06	0.05	0.04	1.48E-04	0.07	0.04	3.47E-04
Coprococcus eutactus	0.06	0.03	1.59E-03	0.05	0.03	1.37E-03	0.11	0.04	9.73E-04
Clostridium polysaccharolyticum	0.18	0.06	5.58E-04	0.18	0.08	8.08E-05	0.23	0.18	3.72E-03
Fusibacter paucivorans	0.13	0.43	8.10E-02	0.14	0.71	1.20E-01	0.26	1.50	3.37E-01
Fusobacterium alocis	1.38	873.17	8.69E-01	0.61	1.07	5.47E-01	0.25	7.48	3.66E-01
Clostridium paradoxum	0.14	0.02	9.83E-05	0.18	0.10	6.80E-03	0.44	0.38	1.10E-01
Clostridium bifermentans	0.55	0.40	1.65E-01	0.32	0.16	1.66E-02	0.59	0.56	2.65E-01
Alicyclobacillus acidocaldarius	0.75	0.25	1.48E-01	0.60	0.21	5.07E-02	0.73	0.15	1.62E-02
Sulfobacillus thermosulfidooxidans	0.29	0.16	1.01E-02	0.35	0.27	4.63E-02	0.44	0.18	1.07E-02
Bacillus spp.	0.91	0.81	8.14E-01	2.78	4.82	1.66E-01	2.55	0.97	9.53E-04
Bacillus sp.	0.16	0.09	2.73E-04	0.26	0.12	1.69E-03	0.31	0.15	6.50E-03
Bacillus senegalensis	1.59	1.50	3.30E-01	3.06	2.02	3.38E-02	1.33	0.53	2.40E-01
L13147 str. B775	0.29	0.37	6.27E-02	0.53	0.70	2.79E-01	0.42	0.21	7.82E-03
Lactobacillus spp.	0.28	0.17	1.26E-02	0.49	0.31	7.70E-02	0.64	0.20	1.19E-02
Lactobacillus spp.	0.92	0.59	7.81E-01	1.74	2.34	3.52E-01	4.24	4.44	3.56E-02
Streptococcus gallolyticus	2.76	1.37	7.52E-03	3.50	1.46	1.39E-03	9.45	3.11	6.73E-05
Acholeplasma oculi	21.05	24.12	6.33E-03	17.51	10.18	1.24E-03	14.46	10.71	3.30E-03
Clostridium ramosum	0.12	0.19	2.81E-02	0.15	0.24	3.92E-02	0.24	0.21	2.20E-02
Clostridium sp.	0.18	0.05	4.72E-04	0.18	0.03	1.75E-05	0.30	0.17	5.90E-03
Clostridium innocuum	0.12	0.09	5.45E-03	0.06	0.12	3.30E-03	1.03	2.88	9.69E-01
AY343175 clone REC6M	0.11	0.19	3.17E-02	0.10	0.28	5.26E-02	0.14	0.29	1.19E-01
AF371739 clone p-4177-6Wa5	0.03	0.02	1.83E-06	0.05	0.36	5.80E-02	0.09	0.06	7.37E-04
Clostridium putrefaciens	0.54	0.48	2.02E-01	1.25	1.21	6.21E-01	1.25	0.54	3.04E-01
Clostridium cellulovorans	0.37	0.14	1.21E-02	0.42	0.05	8.05E-04	0.70	1.15	5.76E-01
AY147280 clone THM-10	0.94	0.67	8.60E-01	0.64	0.19	5.80E-02	1.31	0.64	3.25E-01
Clostridium tetanomorphum	0.58	0.57	2.51E-01	1.02	0.51	9.50E-01	0.96	0.52	8.78E-01
Thermus spp.	0.10	0.06	7.93E-04	0.08	0.03	1.10E-05	0.09	0.11	9.29E-03
Thermus spp.	2.14	6.97	4.03E-01	2.02	2.41	2.30E-01	0.14	6.17	3.41E-01

Appendix B

Table B1: *Genes up-regulated twofold or greater in the neonatal rat GI tract 12 h after feeding E. coli A192PP to P2 pups*

Gene symbol	Description	Function	Mean-fold change
RT1-Aw2	RT1 class Ib, locus Aw2	Antigen presentation	17.71
Fam13a1	family with sequence similarity 13, member A1	Regulation of small GTPase mediated signal transduction	8.45
Malat1	metastasis associated lung adenocarcinoma transcript 1	Non-protein coding regulator of cell motility	6.22
Btg2	BTG family, member 2	Negative regulator of cell proliferation	5.38
Luc7l3	LUC7-like 3	RNA binding regulator of apoptosis	5.26
Setd5	SET domain containing 5	Unknown	5.11
Wdfy1	WD repeat and FYVE domain containing 1	Endosomal trafficking protein	5.08
Eif2c2	eukaryotic translation initiation factor 2C, 2	Regulator of RNA mediated gene silencing	5.05
Pdlim5	PDZ and LIM domain 5	Regulator of cytoskeletal organization	5.05
Cirbp	cold inducible RNA binding protein	Positive regulator of cellular stress response	5.04
Zeb2	zinc finger E-box binding homeobox 2	Negative regulator of cell-cell adhesion	4.89
Sltm	SAFB-like, transcription modulator	Transcriptional inhibitor promoting apoptosis	4.86
Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	Protein ADP ribosylation	4.77
Sv2b	synaptic vesicle glycoprotein 2b	Endocrine cell transmembrane transporter	4.75
Hhip	hedgehog-interacting protein	Negative regulator of angiogenesis	4.64
Mfap3	microfibrillar-associated protein 3	Component of the elastin-associated microfibrils	4.54
Pja2	praja 2, RING-H2 motif containing	Ubiquitin-protein ligase	4.51
Swi5	SWI5 recombination repair homolog	DNA repair complex component	4.50
Mllt10	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog)	Transcription factor	4.39
Slc6a6	solute carrier family 6 (neurotransmitter transporter, taurine)	Taurine transporter	4.31
Evi5	ecotropic viral integration site 5	Regulator of cell cycle and cytokinesis	4.30
Ednrb	endothelin receptor type B	Non-specific receptor for endothelin	4.29
Ddx6	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	RNA degradation in cellular stress response	4.28
Clec2h	C-type lectin domain family 2 member H	Regulator of natural killer cell-mediated cytolysis	4.18
Bcl11b	B-cell CLL/lymphoma 11B (zinc finger protein)	Lymphocyte transcription factor	4.05
Tmed5	transmembrane emp24 protein transport domain	Type I membrane protein, unknown function	4.04
Srrm1	serine/arginine repetitive matrix 1	Spliceosome component	3.96
Xiap	X-linked inhibitor of apoptosis	Apoptotic suppressor	3.91
Cald1	caldesmon 1	Regulator of actin/myosin interactions	3.88
Lpgat1	lysophosphatidylglycerol acyltransferase 1	Catalyzes the reacylation of LPG to phosphatidylglycerol	3.68
Smurf2	SMAD specific E3 ubiquitin protein ligase 2	E3 ubiquitin-protein ligase	3.56
Arid4a	AT rich interactive domain 4A (Rbp1 like)	Transcriptional repressor	3.55
Aff4	AF4/FMR2 family, member 4	Transcription factor	3.47

Gpatch8	G patch domain containing 8	RNA binding protein, unknown function	3.44
Igfbp5	insulin-like growth factor binding protein 5	Regulator of cellular growth factors	3.44
Sptbn1	spectrin, beta, non-erythrocytic 1	Actin-membrane molecular scaffold protein	3.43
Nipbl	nipped-B homolog	DNA repair complex component	3.41
LOC81816	hypothetical protein LOC81816	Putative ubiquitin conjugating enzyme	3..39
N4bp1	Nedd4 binding protein 1	Inhibitor of the E3 ubiquitin-protein ligase ITCH	3.37
Tmem161b	transmembrane protein 161B	Multipass membrane protein, unknown function	3.37
Spnb2	spectrin β2	Actin-membrane molecular scaffold protein	3.31
Atp8b1	ATPase, Class I, type 8B, member 1	Transport of bile acids from intestinal contents to mucosa	3.31
Jarid1a	jumonji, AT rich interactive domain 1A (Rbp2 like)	Histone demethylase regulating cell proliferation	3.28
Pum1	pumilio homolog 1	RNA binding protein regulating cell proliferation	3.28
Dock4	dedicator of cytokinesis 4	Regulator of cell-cell adhesion	3.27
Eif2c1	eukaryotic translation initiation factor 2C, 1	Regulator of RNA mediated gene silencing	3.3
Znf292	zinc finger protein 292	Putative transcriptional regulator	3.26
Sfrs2ip	splicing factor, arginine/serine-rich 2, interacting protein	Regulator of spliceosome assembly	3.24
Eif3c	eukaryotic translation initiation factor 3, subunit C	Initiator of protein synthesis	3.18
Csnk2a1	casein kinase 2, α1 polypeptide	Serine/threonine protein kinase regulating cell proliferation	3.18
Ptprb	protein tyrosine phosphatase, receptor type, B	Signalling protein involved in maintenance of endothelial integrity	3.15
LOC100192313	hypothetical protein LOC100192313	Unknown	3.13
Cmip	c-Maf-inducing protein	Signalling protein involved in Th2 cell activation	3.11
LOC687839	hypothetical protein LOC687839	Unknown function	3.09
Zfp451	zinc finger protein 451	Putative transcriptional regulator	3.05
Tnrc6b	trinucleotide repeat containing 6B	Regulator of RNA mediated gene silencing	3.05
Dek	DEK oncogene	Involved in splice site selection during mRNA processing	3.05
Acbd3	acyl-Coenzyme A binding domain containing 3	Maintenance of Golgi structure	3.04
Rad26l	putative repair and recombination helicase	Putative DNA repair enzyme	3.01
Srpk2	SFRS protein kinase 2	Spliceosome assembly and trafficking of splicing factors	3.00
Hspca	heat shock protein 90α (cytosolic), class A member 1	Molecular chaperone induced by cellular stress	2.98
Slc4a7	solute carrier family 4, sodium bicarbonate co-transporter, member 7	Regulator of intracellular pH	2.96
Loxl2	lysyl oxidase-like 2	Putative role in connective tissue biogenesis	2.94
Laptm4a	lysosomal protein transmembrane 4α	Lysosomal membrane small molecule trafficking	2.91
Rc3h2	ring finger and CCCH-type zinc finger domains 2	Membrane associated DNA binding protein	2.91
Zfp91	zinc finger protein 91	Atypical E3 ubiquitin-protein ligase involved in anti-apoptosis	2.88
Crebl2	cAMP responsive element binding protein-like 2	Cell cycle regulator	2.88
Ankrd11	ankyrin repeat domain 11	Inhibitor of ligand dependent transcriptional activation	2.87
Maf	v-maf musculoaponeurotic fibrosarcoma oncogene homolog	Broad transcriptional regulator, induces T-cell apoptosis	2.86
Igf2r	insulin-like growth factor type 2	Involved in trafficking of lysosomal enzymes and T-cell activation	2.85
Mgll	monoglyceride lipase	Gut epithelial lipase	2.85
Tlk1	tousled-like kinase 1	Nuclear signalling kinase	2.83

Ubxn4	UBX domain protein 4	Involved in endoplasmic reticulum-associated protein degradation	2.83
LOC312273	trypsin V-A	Putative digestive protease	2.82
Dhx36	DEAH (Asp-Glu-Ala-His) box polypeptide 36	Involved in mRNA degradation	2.82
Tpr	translocated promoter region	Involved in nuclear protein import	2.82
Xrn2	5'-3' exoribonuclease 2	RNase, unknown function	2.81
Tns1	tensin1	Focal adhesion component involved in ECM/cytoskeletal interaction	2.80
Creg1	cellular repressor of E1A-stimulated genes 1	Interacts with Igf2r to promote cell growth	2.79
Mga	MAX gene associated	Regulator of cell growth and apoptosis	2.79
Stat3	signal transducer and activator of transcription 3	Transcription factor mediating cytokine receptor signalling pathways	2.77
Rbm9	RNA binding motif protein 9	Regulates splicing of tissue specific exons	2.74
Snrp70	small nuclear ribonucleoprotein 70	Regulator of pre-mRNA splicing	2.72
Slc44a1	solute carrier family 44, member 1	Choline transporter involved in membrane sysnthesis	2.71
Dag1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	Extracellular matrix receptor	2.71
Ubn1	ubinuclein 1	Regulator of cell death	2.69
Eif4g1	eukaryotic translation initiation factor 4γ 1	Involved in mRNA recruitment to ribosome	2.69
Gcap14	granule cell antiserum positive 14	Unknown function	2.68
Mobkl1a	MOB1, Mps One Binder kinase activator-like 1A	Regulator of cell growth and apoptosis	2.68
Arhgef12	Rho guanine nucleotide exchange factor (GEF) 12	Regulator of RhoA GTpase activity	2.68
Reg3b	regenerating islet-derived 3β	Antimicrobial peptide with C-type lectin domain	2.63
Kcnma1	calcium-activated channel, subfamily Mα	Calcium ion activated potassium channel	2.63
Cbl	Cas-Br-M ecotropic retroviral transforming sequence	Involved in signal transduction in hematopoietic cells	2.62
Rnd3	Rho family GTPase 3	Regulator of actin cytoskeletal organization	2.61
Sox4	SRY-box 4	Transcriptional activator involved in development	2.61
Pik3r1	phosphoinositide-3-kinase, regulatory subunit 1α	Adaptor mediating association of activated kinases to the plasma membrane	2.60
Hoxb6	homeobox B6	Transcriptional regulator	2.58
Bend7	BEN domain containing 7	Unknown	2.57
Arhgap5	Rho GTPase activating protein 5	Regulator of actin cytoskeletal organization	2.56
Eml4	echinoderm microtubule associated protein like 4	Putative role in microtubule assembly dynamics	2.56
Ralgps2	Ral GEF with PH domain and SH3 binding motif 2	Putative role in cytoskeletal organization	2.55
Falz	fetal Alzheimer antigen	Histone binding component of nucleosome-remodelling factor	2.55
Il6st	interleukin 6 signal transducer	Intracellular transducer of cytokine signalling	2.55
Otub1	OTU domain, ubiquitin aldehyde binding 1	Ubiquitin hydrolase regulating T-cell anergy	2.54
Topbp1	topoisomerase (DNA) II binding protein 1	Regulator of DNA damage response	2.54
Slc4a4	solute carrier family 4 (anion exchanger), member 4	Regulator of intracellular pH	2.54
Thoc2	THO complex 2	Involved in mRNA export	2.53
Hsp90ab1	heat shock protein 90kDa α (cytosolic), class B member 1	Molecular chaperone involved in cellular stress response	2.52
Itgb3	integrin β3	Mediates cellular adhesion to ECM	2.52
Rps6ka5	ribosomal protein S6 kinase, polypeptide 5	Kinase required for activation of stress-induced transcription factors	2.51
Thra	thyroid hormone receptor α	Nuclear hormone receptor	2.51
Ccar1	cell division cycle and apoptosis regulator 1	Regulator of cellular proliferation	2.51

Zdhhc20	zinc finger, DHHC-type containing 20	Unknown	2.50
Clcn5	chloride channel 5, transcript variant 6	Acidification of the endosomal lumen	2.50
Cisd2	CDGSH iron sulfur domain 2	Regulator of autophagy	2.50
Lcorl	ligand dependent nuclear receptor co-repressor-like	Transcriptional regulator	2.49
Ccnd2	cyclin D2	Cell cycle regulator	2.48
LOC100363275	G protein-coupled receptor 124	Unknown	2.48
Itsn2	intersectin 2	Involved in T-cell receptor endocytosis	2.46
Samd8	sterile alpha motif domain containing 8	Unknown	2.46
Ubn2	ubinuclein 2	Regulator of cell death	2.46
Bmpr2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	Involved in calcium regulation	2.45
Sf3b2	splicing factor 3b, subunit 2	Subunit of splicing factor SF3B	2.44
LOC681371	hypothetical protein LOC681371	Unknown function	2.44
Narg1	NMDA receptor regulated 1	Acetyltransferase involved in hematopoeitic and neuronal development	2.44
Strn3	striatin, calmodulin binding protein 3	Signalling or scaffolding protein involved in modulating calmodulin activity	2.44
Pa2g4	proliferation-associated 2G4	Regulator of cell proliferation	2.44
Srrm2	serine/arginine repetitive matrix 2	Involved in pre-mRNA splicing	2.42
Fermt2	fermitin family homolog 2	Participates in actin organization and cytoskeletal-ECM adhesion	2.41
Chd1	chromodomain helicase DNA binding protein 1	Chromatin remodelling	2.40
Tcf4	transcription factor 4	Enhancer of immunoglobulin expression	2.40
Ankle2	ankyrin repeat and LEM domain containing 2	Unknown function	2.40
Trio	triple functional domain (PTPRF interacting)	Involved in cytoskeletal rearrangement	2.39
Herc1	hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	Regulator of membrane trafficking	2.38
Vdac1	voltage-dependent anion channel 1	Mitochondrial membrane channel involved in apoptosis	2.38
LOC286960	preprotrypsinogen IV	Trypsin-like serine protease	2.38
Hectd1	HECT domain containing 1	Ubiquitin-protein ligase	2.38
Rbm25	RNA binding motif protein 25	Splicing regulator involved in apoptosis	2.37
Clk1	CDC-like kinase 1	Putative regulator of RNA splicing	2.37
Nfix	nuclear factor I/X (CCAAT-binding transcription factor)	Transcriptional activator	2.36
Wasl	Wiskott-Aldrich syndrome-like	Regulator of actin polymerization	2.36
Ash1l	ash1 (absent, small, or homeotic)-like	Histone methyltransferase	2.36
Traf6	Tnf receptor-associated factor 6	Ubiquitin ligase responsible for activating NFκB after IL-1 receptor signalling	2.36
Marcks	myristoylated alanine rich protein kinase C substrate	F-actin cross-linker	2.36
Rps6ka1	ribosomal protein S6 kinase polypeptide 1	Mediator of stress-induced transcriptional activation	2.35
RT1-CE12	RT1 class I, locus CE12	Antigen presentation	2.35
Cpd	carboxypeptidase D	Regulatory peptidase involved in NO synthesis during inflammation	2.35
Wbp4	WW domain binding protein 4 (formin binding protein 21)	Promotes pre-mRNA splicing	2.34
LOC685707	similar to neuron navigator 1	Similar to protein regulating neuronal development	2.34
Nktr	natural killer tumor recognition protein	NK-cell receptor	2.33

U2af1	U2 small nuclear ribonucleoprotein auxiliary factor	Involved in mRNA splicing	2.32
Ptch1	patched 1	Hedgehog gene receptor	2.32
Pgap2	post-GPI attachment to proteins 2	Involved in anchoring proteins to the plasma membrane	2.31
Zc3h11a	zinc finger CCCH-type containing 11A	Unknown function	2.31
Raph1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	Negatively regulates cell adhesion	2.31
Mpp6	membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)	Regulator of membrane receptor clustering	2.30
Nr2f2	nuclear receptor subfamily 2, group F, member 2	Steroid thyroid hormone receptor	2.29
Pkp4	plakophilin 4	Regulator of cadherin function	2.29
Id3	inhibitor of DNA binding 3	Inhibitor of transcription factor DNA binding	2.29
Zdhhc21	zinc finger, DHHC domain containing 21	Unknown function	2.29
Ppp1r12a	protein phosphatase 1, regulatory (inhibitor) subunit 12A	Regulates myosin phosphatase activity	2.28
Pafah1b1	platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	Required for proper activation of Rho GTPases and actin polymerization	2.28
Rbm5	RNA binding motif protein 5	Component of the spliceosome A complex	2.28
Lin7	lin-7 homolog C	Involved in maintaining cellular polarity	2.28
Trim39	tripartite motif containing 39	Inhibits proteosomal degradation of pro-apoptotic factors	2.27
Brd8	bromodomain containing 8	Co-activiator of nuclear hormone receptors	2.27
Ap4e1	adapter-related protein complex 4 subunit ε-1	Involved in targeting to the endosomal/lysosomal system	2.27
Lrrc8a	leucine rich repeat containing 8 family, member A	Involved in promoting B-cell maturation	2.27
Zfp106	zinc finger protein 106	Unknown function	2.27
Adipor2	adiponectin receptor 2	Involved in lipid metabolic regulation	2.26
Fam126b	family with sequence similarity 126	Unknown function	2.26
Add3	adducin 3γ	Calmodulin binding promoter of actin-spectrin network assembly	2.26
Adrbk2	adrenergic receptor kinase β2	Regulator of receptor function	2.26
Cdh22	cadherin 22	Calcium dependent cell adhesion protein	2.26
Alox15	arachidonate 15-lipoxygenase	involved in the production and metabolism of fatty acid hydroperoxidases	2.25
LOC498544	hypothetical protein LOC498544	Unknown function	2.25
Rcor1	REST corepressor 1	Chromatin remodelling	2.24
Mef2a	myocyte enhancer factor 2a	Activator of numerous growth factor and stress-induced genes	2.23
Dnmt3a	DNA (cytosine-5-)-methyltransferase 3α	DNA methylation	2.23
Vezf1	vascular endothelial zinc finger 1	Regulation of IL-3 expression	2.23
Eif5	eukaryotic translation initiation factor 5	Initiator of protein synthesis	2.22
Pip5k2a	phosphatidylinositol-5-phosphate 4-kinase, type IIα	Involved in the regulation of secretion, cell proliferation, differentiation, and motility	2.22
Gatad2b	GATA zinc finger domain containing 2B	Transcriptional repressor	2.21
Ctdspl	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like	Negative regulator of transcription	2.20
Rnf6	ring finger protein (C3H2C3 type) 6	Ubiquitin-protein ligase	2.20
Msi2	Musashi homolog 2	RNA-binding protein regulating cellular proliferation	2.20
Mxd1	MAX dimerization protein 1	Regulator of cellular proliferation and apoptosis	2.19

Arl4d	ADP-ribosylation factor-like 4D	Involved in intracellular membrane trafficking	2.18
Krt15	keratin 15	Responsible for the structural integrity of epithelial cells	2.18
Mll5	myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog)	Histone methyltransferase	2.17
Rbm27	RNA binding motif protein 27	Unknown function	2.17
Ptprs	protein tyrosine phosphatase, receptor type, S	Signalling protein involved in development	2.17
Entpd5	ectonucleoside triphosphate diphosphohydrolase 5	Regulator of ATP usage	2.16
Eif4a1	eukaryotic translation initiation factor 4A1	RNA-helicase allowing mRNA-ribosome interaction	2.15
Phc3	polyhomeotic-like 3	Involved in transcriptional repression	2.15
Frg1	FSHD region gene 1	Involved in processing pre-rRNA	2.15
Eif5b	eukaryotic translation initiation factor 5B	Promotes binding of methionine-tRNA to ribosome	2.15
Hmox1	heme oxygenase (decycling) 1	Essential enzyme in heme metabolism	2.14
Trove2	TROVE domain family, member 2	Regulator of Y-RNAs	2.13
Sox11	SRY-box containing gene 11	Developmental regulator	2.13
Arid4b	AT rich interactive domain 4B (Rbp1 like)	Transcriptional repressor	2.12
Mphosph8	M-phase phosphoprotein 8	Involved in cell-cycle	2.12
LOC688495	hypothetical protein LOC688495	Unknown function	2.11
Rgs4	regulator of G-protein signalling 4	Negative regulator of G-protein signaling	2.11
Meg3	maternally expressed 3	Regulator of cell proliferation	2.11
Rbm39	RNA binding motif protein 39	Involved in mRNA splicing	2.11
Trak2	trafficking protein, kinesin binding 2	Regulator of endosome to lysosome trafficking	2.10
Ahi1	Abelson helper integration site 1	Involved in neuronal development	2.10
Akap13	A kinase (PRKA) anchor protein 13	Anchors cAMP-dependent kinase	2.10
Wdr37	WD repeat domain 37	Regulator of signal transduction and apoptosis	2.09
Lgr4	leucine-rich repeat containing G protein-coupled receptor 4	Orphan receptor	2.09
C-myb	Myb proto-oncogene	Regulates differentiation of hematopoeitic cells	2.09
Sfrs11	splicing factor, arginine/serine-rich 11	Involved in pre-mRNA splicing	2.08
Rdx	radixin	Binds barbed ends of actin filaments to cell membrane	2.07
Stk25	serine/threonine kinase 25 (STE20 homolog)	Stress-activated kinase regulating protein export and cell adhesion	2.06
Rhoj	ras homolog gene family, member J	Regulates cell morphology via increased F-actin formation	2.06
Rod1	ROD1 regulator of differentiation 1	Regulator of cell differentiation	2.06
Ppap2b	phosphatidic acid phosphatase type 2B	Involved in cell adhesion and cell-cell interactions	2.06
Tsc22d4	TSC22 domain family, member 4	Transcriptional repressor	2.05
Arglu1	arginine and glutamate rich 1	Unknown function	2.05
Arid3a	AT rich interactive domain 3A (Bright like)	Transcription factor involved in B-cell differentiation	2.05
Rad54l2	Rad54 like 2	DNA helicase	2.04
Ppargc1b	peroxisome proliferator-activated receptor gamma, co-activator 1β	Involved in fat oxidation and non-oxidative glucose metabolism	2.04
Scn7a	sodium channel, voltage-gated, type VIIα	Mediates sodium ion permeability of membranes	2.04
Brd4	bromodomain containing 4	Chromatin remodelling	2.04
Fnip2	folliculin interacting protein 2	Signal transducer of pro-apoptotic factors	2.04
Wbp4	WW domain binding protein 4 (formin binding protein 21)	Involved in pre-mRNA splicing	2.04

Esf1	ESF1, nucleolar pre-rRNA processing protein, homolog	Transcriptional regulator	2.04
Zc3h12c	zinc finger CCCH type containing 12C	Putative RNase	2.04
Luc7l2	Luc7-like 2	Unknown function	2.03
Eef1a1	eukaryotic translation elongation factor 1α 1	Promoter of protein biosynthesis	2.03
Rock2	Rho-associated coiled-coil containing protein kinase 2	Regulates actin assembly	2.03
Lsm12	LSM12 homolog	Unknown function	2.02
Nolc1	nucleolar and coiled-body phosphoprotein 1	Involved in RNA polymerase I catalysed transcription	2.02
Safb	scaffold attachment factor B	Anchor for RNA polymerase II transcriptomal complex	2.02
Chka	choline kinase α	Involved in phospholipid biosynthesis	2.01
Polr3k	polymerase (RNA) III (DNA directed) polypeptide K	RNA polymerase III component	2.01
Sms	spermine synthase	Involved in polyamine metabolism	2.01
Axin2	axin 2	Regulates Wnt signalling by interaction with β-catenin	2.00
Fatp4	fatty acid transport protein 4	Transport of long chain fatty acids	2.00
Trps1	trichorhinophalangeal syndrome I homolog	Transcriptional regulator of columnar cell differentiation	2.00

Table B2: *Genes down-regulated twofold or greater in the neonatal rat GI tract 12 h after feeding E. coli A192PP to P2 pups.*

Gene symbol	Description	Function	Mean-fold change
Tff2	trefoil factor 2	Defence of the mucosal barrier	-24.63
Mgam	Maltase-glucoamylase, intestinal	Brush border hydrolase	-5.49
RGD:727924	rRNA promoter binding protein	Regulator of cell proliferation	-4.33
Ins2	insulin 2	Hormone regulating carbohydrate and fat metabolism	-3.97
RT1-A3	RT1 class I, locus A3	Antigen presentation	-3.11
RT1-CE15	RT1 class I, locus CE15	Antigen presentation	-2.86
Wtap	Wilms tumor 1 associated protein	Transcriptional and post-transcriptional regulator	-2.65
Senp5	Sumo1/sentrin/SMT3 specific peptidase 5	Protease involved in cell division	-2.65
Ins1	insulin 1	Hormone regulating carbohydrate and fat metabolism	-2.64
Mcpt3	mast cell peptidase 3	Serine endopeptidase	-2.62
Ubd	ubiquitin D	Targeting for proteosomal degradation	-2.48
RT1-Db1	RT1 class II, locus Db1	Antigen presentation	-2.46
Pbx1	pre-B-cell leukemia transcription factor	Transcriptional regulator	-2.36
Pim1	pim-1 oncogene	Signalling kinase activity	-2.35
Dcaf12	DDB1 and CUL4 associated factor 12	Regulation of ligase activity	-2.34
Mcpt4	mast cell protease 4	Serine endopeptidase	-2.30
LOC100362483	H2-GS14-2 antigen	Regulation of antigen presentation	-2.29
Birc6	baculoviral IAP repeat containing 6	Regulation of apoptosis	-2.27
ABCB10	ATP binding cassette family	Membrane transporter	-2.26
Mcpt1	mast cell protease 1	Serine endopeptidase	-2.26
Coro1c	coronin, actin binding protein 1C	Involved in cytokinesis	-2.23
Itgav	integrin αV	Extracellular matrix receptor	-2.22

Actn4	actinin α4	Intracellular actin anchoring	-2.20
Fam100b	family with sequence similarity 100, member B	Unknown	-2.16
Wwc1	WW and C2 domain containing 1	Transcriptional activator	-2.15
Gnptab	N-acetylglucosamine-1-phosphate transferase	Regulator of lysosomal transport	-2.13
Nfkbil1	Ikb family protein	NFκB inhibitor relative	-2.09
Slc1a3	solute carrier family 1, member 3	Glutamate transporter	-2.05
Spag9	sperm associated antigen 9	Regulator of MAPK cascade	-2.05
Larp1	La ribonucleoprotein domain family, member 1	RNA degradation	-2.05
Pga5	pepsinogen 5, group I	Digestive protease	-2.04
Dusp6	dual specificity phosphatase 6	Regulator of MAPK cascade	-2.04
Coa5	cytochrome C oxidase assembly factor 5	mitochondrial complex IV assembly	-2.04
Amy1 ; Amy2	amylase α1A (salivary), amylase 2, pancreatic	Hydrolase	-2.03
Socs2	suppressor of cytokine signalling 2	Regulator of cell signalling	-2.02
Daf1	Cd55 molecule	Classical complement pathway activator	-2.02

Table B3: *Genes up-regulated twofold or greater in the neonatal rat GI tract 12 h after feeding E. coli A192PP to P9 pups*

Gene Symbol	Description	Function	Mean-fold change
RT1-Bb	RT1 class II, locus Bb	Antigen presentation	11.77
Ints7	Integrator complex subunit 7	Involved in mRNA processing	5.53
Defa-rs1	defensin α-related sequence 1	α-defensin-type antimicrobial peptide	5.44
Cirbp	cold inducible RNA binding protein	Positive regulator of cellular stress response	5.21
Pdcd4	programmed cell death 4	Inhibitor of protein biosynthesis	5.08
Cct6a	chaperonin containing Tcp1, subunit 6A ξ1	Chaperone involved in correct actin and tubulin folding	4.89
Sept2	septin 2	Filament forming cytoskeletal GTPase	4.86
RT1-CE15	RT1 class I, locus CE15	Antigen presentation	4.45
St6gal1	ST6 beta-galactosamide α-2,6-sialyltranferase 1	Transfers sialic acid to galactose containing receptor substrates	4.41
Clic4	chloride intracellular channel 4	Membrane associated ion channel	4.36
Tm9sf3	transmembrane 9 superfamily member 3	Unknown function	4.11
RT1-Aw2	RT1 class Ib, locus Aw2	Antigen presentation	3.40
Casp3	caspase 3	Effector caspase mediating apoptosis	3.95
Caprin1	cell cycle associated protein 1	Regulation of mRNA transport	3.95
Vsig10l	V-set and immunoglobulin domain containing 10 like	Unknown function	3.82
Hnrnpa2b1	heterogeneous nuclear ribonucleoprotein A2/B1	Involved in mRNA processing	3.67
Scgb1a1	secretoglobin, family 1A, member 1 (uteroglobin)	Anti-inflammatory regulator	3.63
Vcl	vinculin	Actin binding protein involved in cell-cell and cell-ECM adhesion	3.60
Nid1	nidogen 1	Laminin-associated protein involved in cell-ECM adhesion	3.59
Mylk	myosin light chain kinase	Regulator of actin-myosin interaction	3.55
Gatad2b	GATA zinc finger domain containing 2B	Transcriptional repressor	3.53
Hnf4a	hepatocyte nuclear factor 4α	Transcription factor regulating development	3.43

Car3	carbonic anhydrase 3	Reversible hydration of carbon dioxide	3.40
Actr3	ARP3 actin-related protein 3 homolog	ARP2/3 complex component, involved in cell motility	3.38
Prkacb	protein kinase, cAMP dependent, catalytic, β	Mediates cAMP-dependent signalling	3.38
RGD1309534	Similar to RIKEN cDNA 4931406C07	Unknown function	3.38
Foxn3	forkhead box N3	Transcriptional repressor responding to DNA damage	3.36
Ssr3	signal sequence receptor γ	Regulator of protein-ER attachment	3.34
Pak2	p21 protein (Cdc42/Rac)-activated kinase 2	Apoptotic regulator	3.32
Cav1	caveolin 1, caveolae protein	Co-stimulator of T-cell receptor mediated T-cell activation	3.30
Gna11	guanine nucleotide binding protein α11	Transmembrane signalling transducer	3.30
Mat2a	methionine adenosyltransferase IIα	Catalyses the production of S-adenosylmethionine	3.28
Tgoln1	trans-golgi network protein	Unknown function	3.27
Cav2	caveolin 2	Major component of plasma membrane caveolae	3.17
Ppm1a	protein phosphatase 1A, magnesium dependent, α isoform	Negative regulator of cellular stress response	3.16
Pigt	phosphatidylinositol glycan anchor biosynthesis, class T	Involved in GPI cell surface protein anchor biosynthesis	3.15
Golph3	Golgi phosphoprotein 3 (coat-protein)	Regulator of Golgi trafficking	3.11
Defa24	defensin 24α	α-defensin-type antimicrobial peptide	3.09
Hsd3b7	hydroxy-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 7	Involved in hormonal steroid biosynthesis	3.06
Canx	calnexin	Molecular chaperone ensuring correct glycoprotein folding	3.05
Crk	v-crk sarcoma virus CT10 oncogene homolog	Involved in phagocytosis of apoptotic cells	3.03
Il13ra1	interleukin 13 receptor 1α	IL-13 and IL-4 receptor	3.01
Eif1a	eukaryotic translation initiation factor 1A	Promotes accurate ribosomal assembly	3.01
Rab5b	RAB5B, member RAS oncogene family	Involved in vesicular trafficking	3.00
Lin7c	lin-7 homolog C	Involved in maintenance of cellular polarity	2.98
Cbfb	core-binding factor, β subunit	Broad transcriptional regulator	2.95
Rcc2	regulator of chromosome condensation 2	Involved in cytokinesis	2.94
Tmem47	transmembrane protein 47	Unknown function	2.94
Rnf114	ring finger protein 114	Unknown function	2.93
Cd36	CD36 molecule (thrombospondin receptor)	Involved in cell adhesion and fatty acid transport	2.93
App	amyloid β (A4) precursor protein	Involved in neuronal growth	2.91
Zfp68	zinc finger protein 68	Unknown function	2.91
LOC683399	region containing similar to NGF-binding Ig light chain	Unknown function	2.90
Gpbp1	GC-rich promoter binding protein 1	Transcriptional regulator	2.89
Mcam	melanoma cell adhesion molecule	Involved in cell adhesion	2.88
LOC683788	similar to Fascin (Singed-like protein)	Unknown function	2.86
Gga2	Golgi associated, γ adaptin ear containing, ARF binding protein 2	Regulator of endosomal-lysosomal trafficking	2.86
Rod1	ROD1 regulator of differentiation 1	Involved in cellular differentiation	2.85
Stat5b	signal transducer and activator of transcription 5B	IL-2 and IL-4 signal transducer	2.78
Prcp	prolylcarboxypeptidase (angiotensinase C)	Lysosomal prolylcarboxypeptidase	2.78

Prkci	protein kinase Cí	Involved in formation of epithelial tight junctions	2.78
Lbr	lamin B receptor	Anchors laminin and heterochromatin to the nuclear membrane	2.77
Pik3r1	phosphoinositide-3-kinase, regulatory subunit 1α	Adaptor mediating protein-tyr kinase membrane binding	2.74
Tmem45b	transmembrane protein 45b	Unknown function	2.73
Txnrd1	thioredoxin reductase 1	Involved in protection from oxidative stress	2.72
Atp1a1	ATPase, Na+/K+ transporting, α1 polypeptide	Regulator of membrane electrochemical gradients	2.72
Lasp1	LIM and SH3 protein 1	Regulator of dynamic actin formation	2.72
Ankle2	ankyrin repeat and LEM domain containing 2	Unknown function	2.70
Gnai3	guanine nucleotide binding protein (G protein), α inhibiting 3	Modulator of trans-membrane signalling systems	2.70
Rtn4	reticulon 4	Inhibitor of Bcl-xl and Bcl-2 anti-apoptotic activity	2.70
Col6a3	procollagen, type VIα 3	Cell binding protein	2.68
Krt15	keratin 15	Epithelial structural integrity	2.66
RGD1306148	similar to KIAA0368	Unknown function	2.65
Picalm	phosphatidylinositol binding clathrin assembly protein	Involved in clatherin coated pit formation	2.65
Cxcl12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	T-cell and monocyte chemoattractant	2.65
Pank3	pantothenate kinase 3	Regulator of CoA biosynthesis	2.65
Myh11	myosin, heavy chain 11, smooth muscle	Involved in smooth muscle contraction	2.64
Ocln	occludin	Involved in formation and regulation of epithelial tight junctions	2.64
Galnt1	N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	Catalyses O-linked oligosaccharide formation	2.64
Akirin2	akirin 2	Downstream effector of cytokine signalling	2.63
Fnbp1l	formin binding protein 1-like	Involved in actin reorganization during endocytosis	2.62
Stard5	StAR-related lipid transfer (START) domain containing 5	Involved in intracellular transport of sterols and other lipids	2.62
Far1	fatty acyl CoA reductase 1	Catalyzes the reduction of saturated fatty acyl-CoA	2.62
S100a6	S100 calcium binding protein A6	Calcium sensor involved in cellular differentiation	2.62
Ptprs	protein tyrosine phosphatase, receptor type, S	Transmembrane signalling transducer	2.61
Zyg11b	zyg-ll homolog B	E3 ubiquitin-ligase complex component	2.61
Hspa2	heat shock protein 2α	Stress-induced molecular chaperone	2.61
Slc5a1	solute carrier family 5 (sodium/glucose cotransporter), member 1	Mediates glucose/galactose uptake from intestinal lumen	2.61
Rbpj	recombination signal binding protein for immunoglobulin κ J region	Transcriptional regulator of NOTCH (cell-cell) signalling	2.59
Rab31	RAB31, member RAS oncogene family	Involved in vesicle and granule targeting	2.58
Eif3s6ip	eukaryotic translation initiation factor 3, subunit 6 interacting protein	Initiator of protein synthesis	2.58
Smtn	smoothelin	Stress fibre cytoskeletal component	2.58
Arl2bp	ADP-ribosylation factor-like 2 binding protein	Regulator of STAT activity	2.57
Ireb2	iron responsive element binding protein 2	Regulator of ferretin/transferrin expression	2.57
Nov	nephroblastoma over-expressed gene	Regulator of cell growth	2.55
Stk17b	serine/threonine kinase 17b	Positive regulator of apoptosis	2.55
Ppp2r4	protein phosphatase 2A activator, regulatory subunit 4	Involved in apoptosis and negative regulation of cell growth	2.55
Cap1	CD40 associated protein 1	Inhibitor of NFκB activation	2.55

Tmed2	transmembrane emp24 domain trafficking protein 2	Involved in vesicular trafficking	2.55
Calm3	calmodulin 3	Calcium binding regulator of inflammation, apoptosis and muscle contraction	2.54
Fstl1	follistatin-like 1	Involved in cellular differentiation	2.53
Hoxb13	homeo box B13	Transcription factor regulating development	2.53
Actr2	ARP2 actin-related protein 2 homolog	Involved in actin polymerization	2.53
Id3	inhibitor of DNA binding 3	Regulator of transcription factor function	2.53
Xiap	X-linked inhibitor of apoptosis	Apoptotic suppressor	2.52
Efna1	ephrin A1	Regulator of angiogenesis	2.52
Scamp2	secretory carrier membrane protein 2	Involved in post-Golgi trafficking to the surface membrane	2.52
Ctnnb1	catenin (cadherin associated protein) 1β	Structural component of adherent junctions, and regulator of Wnt responsive genes	2.51
Casp2	caspase 2	Initiator caspase mediating apoptosis	2.51
Jak2	Janus kinase 2	Cytokine receptor signal transducer	2.51
Myh9	myosin, heavy chain 9, non-muscle	Involved in cytokinesis	2.50
Gng2	guanine nucleotide binding protein (G protein) 2γ	Modulator of trans-membrane signalling systems	2.50
Cdx1	caudal type homeo box 1	Regulator of enterocyte differentiation	2.50
Plekhb2	pleckstrin homology domain containing, family B (evectins) member 2	Unknown function	2.50
Ddx3x	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	Helicase involved in interferon response	2.49
Rnf6	ring finger protein (C3H2C3 type) 6	Ubiquitin-protein ligase	2.49
Ap3d1	adaptor-related protein complex 3 1Δ subunit	Involved in intracellular granule trafficking	2.47
Elovl5	ELOVL family member 5, elongation of long chain fatty acids	Involved in elongation of long-chain fatty acids	2.47
Rab5a	RAB5A, member RAS oncogene family	Promotes membrane-endosomal fusion	2.47
Asah1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	Hydrolyzes the sphingolipid ceramide to sphingosine (signalling lipid) and fatty acid	2.46
Kitlg	KIT ligand	Stimulates proliferation of Mast cells	2.46
Arpc2	actin related protein 2/3 complex, subunit 2	Actin binding component of Arp2/3 complex	2.46
Hsph1	heat shock 105kDa/110kDa protein 1	Prevents aggregation of denatured proteins during cellular stress	2.46
Tle4	transducin-like enhancer of split 4 (E(sp1) homolog	Transcriptional co-repressor	2.46
Cdc42se2	CDC42 small effector 2	Involved in actin organization during phagocytosis	2.46
Eif2s3x	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	Involved in protein biosynthesis	2.46
Pfkm	phosphofructokinase, muscle	Regulator of glycolysis	2.45
Dck	deoxycytidine kinase	Phosphorylates deoxynucleotides	2.45
Csnk1a1	casein kinase 1 1α	Participates in Wnt signalling	2.44
Nedd4	neural precursor cell expressed, developmentally down-regulated 4	Ubiquitin-protein ligase	2.44
Slco2b1	solute carrier organic anion transporter family, member 2b1	Organic ion uptake	2.44
Prss35	Protease, serine, 35	Unknown function	2.43
Slc31a1	solute carrier family 31 (copper transporters), member 1	Copper uptake	2.43
Adam10	ADAM metallopeptidase domain 10	Cleaves membrane bound TNF-alpha precursor to its mature form	2.43
Cdkn2b	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	Effector of TGF-beta induced cell-cycle arrest	2.42

Sptlc1	serine palmitoyltransferase, long chain base subunit 1	Key enzyme in sphingolipid synthesis	2.42
Rnf4	ring finger protein 4	Ubiquitin-protein ligase	2.42
Cacybp	calcyclin binding protein	Involved in calcium-dependent ubiquitination	2.42
Tmprss8	transmembrane protease, serine 8 (intestinal)	Unknown function	2.41
Patl1	protein associated with topoisomerase II homolog 1	Involved in RNA degradation	2.41
Sesn3	sestrin 3	Involved in cellular stress response	2.41
Cfh	complement factor H	Regulator of complement activation and microbial specificity	2.40
Tpm1	tropomyosin 1α	Regulator of actin mechanics	2.40
Tspan2	tetraspanin 2	Mediator of trans-membrane signalling systems	2.40
Ahcyl1	adenosylhomocysteinase-like 1	Unknown function	2.40
Tgfbr2	transforming growth factor β receptor II	Receptor inducing apoptosis and negatively regulating phagocyte activation	2.40
Scarf2	scavenger receptor class F, member 2	Involved in cell adhesion	2.39
Ipo5	importin 5	Involved in nuclear protein import	2.39
Sept7	septin 7	Involved in actin cytoskeletal organization	2.39
LOC100363366	amyloid β (A4) precursor-like protein 2-like	Unknown function, interacts with MHC class I molecules	2.38
Dazap2	DAZ associated protein 2	Involved in TGF-beta signalling and stress granule formation	2.38
Rbm9	RNA binding motif protein 9	Regulator of alternative exon splicing	2.38
Drg1	developmentally regulated GTP binding protein 1	May play a role in cell proliferation, differentiation and death	2.38
Slc30a9	solute carrier family 30 (zinc transporter), member 9	Involved in activation of Wnt responsive genes	2.38
Pfn2	profilin 2	Regulator of actin polymerization	2.38
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	Transcriptional regulator	2.38
Cd44	Cd44 molecule	Hyaluronic acid (ECM) receptor, involved in lymphocyte activation	2.37
Efnb1	ephrin B1	Involved in cell adhesion	2.37
Klc1	kinesin light chain 1	Involved in organelle transport	2.36
Kctd12	potassium channel tetramerisation domain containing 12	GABA-B receptor subunit	2.36
Nolc1	nucleolar and coiled-body phosphoprotein 1	Involved in RNA polymerase I transcription	2.36
Pgrmc2	progesterone receptor membrane component 2	Putative steroid receptor	2.36
Vezf1	vascular endothelial zinc finger 1	Transcription factor regulating cell differentiation	2.36
Reep6	receptor accessory protein 6	Unknown function	2.35
Atp2b4	ATPase, Ca++ transporting, plasma membrane 4	Regulator of intracellular calcium homeostatis	2.35
Lgr4	leucine-rich repeat-containing G protein-coupled receptor 4	Orphan receptor	2.35
Pdlim7	PDZ and LIM domain 7	Invovled in actin cytoskeletal organization	2.35
Bid	BH3 interacting domain death agonist	Pro-apoptotic mediator inducing cytochrome c release and inhibiting Bcl-2 activity	2.34
Soat1	sterol O-acyltransferase 1	Involved in lipoprotein assembly and cholesterol absorption	2.34
Gtf2h1	general transcription factor IIH, polypeptide 1	Involved in nucleotide excision repair during transcription	2.34
Mbnl2	muscleblind-like 2	Mediates pre-mRNA splicing regulation	2.34
Sesn1	sestrin 1	Involved in the reduction of peroxiredoxins	2.34
Prkar2a	protein kinase, cAMP dependent regulatory, type IIα	Involved in membrane association of MAP2 kinase	2.33
Atp2a3	ATPase, Ca++ transporting, ubiquitous	Transports calcium from the cytosol to the endoplasmic reticulum	2.32
Nfyc	nuclear transcription factor-Yγ	Regulator of transcription at CCAAT enhancer	2.31

Pkn2	protein kinase N2	Inhibits Akt induced anti-apoptotic activity	2.31
Pi4k2b	phosphatidylinositol 4-kinase type 2β	Regulator of vesicular trafficking	2.31
Gdi2	GDP dissociation inhibitor 2	Involved in vesicular trafficking	2.31
Larp4	La ribonucleoprotein domain family, member 4	Unknown function	2.30
Prim1	DNA primase, p49 subunit	Component of DNA polymerase which synthesizes small Okazaki fragment primers	2.30
Tpm4	tropomyosin 4	Regulator of myosin-actin interactions	2.29
Cdc26	cell division cycle 26	Ubiquitin-ligase involved in cell cycle	2.29
LOC501268	nidogen 2	Basement membrane component involved in adhesion and apoptosis	2.29
Tm9sf4	transmembrane 9 superfamily protein member 4	Unknown function	2.28
Tfrc	transferrin receptor	Mediator of iron uptake	2.27
Bhlhe40	basic helix-loop-helix family, member e40	Involved in control of cell differentiation	2.27
Reg3b	regenerating islet-derived 3β	Antimicrobial peptide with C-type lectin domain	2.27
Tollip	toll interacting protein	Negative regulator of NFκB activation by IL-1 pathway	2.26
Cd3e	CD3 molecule, epsilon polypeptide	Involved in coupling antigen recognition to intracellular signalling pathways	2.26
Rfc1	replication factor C (activator 1) 1	Involved in DNA replication and repair	2.26
Arl8b	ADP-ribosylation factor-like 8B	Involved in lysosomal motility	2.26
Oaz2	ornithine decarboxylase antizyme 2	Regulator of polyamine synthesis	2.26
LOC690372	similar to U2 (RNU2) small nuclear RNA auxiliary factor 2 isoform b	Unknown function	2.26
Slc9a3r1	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	Involved in regulating interactions between cytoskeleton and membrane proteins	2.25
Leprot	leptin receptor overlapping transcript	Decreases cellular response to leptin hormone	2.25
Med14	mediator complex subunit 14	Involved in regulation of RNA polymerase II transcription	2.24
Toe1	target of EGR1, member 1 (nuclear)	Positive regulator of TGF-beta expression	2.24
Cd55	Cd55 molecule	Negative regulator of the complement cascade	2.24
Mgea5	Meningioma expressed antigen 5 (hyaluronidase)	Glycosidase that removes O-GlcNAc from glycoproteins	2.24
Fyttd1	forty-two-three domain containing 1	Involved in mRNA export	2.24
Pla2g10	phospholipase A2, group X	Regulator of cellular lipid content	2.24
Cebpg	CCAAT/enhancer binding protein (C/EBP), gamma	Positive regulator of IL-4 expression	2.23
Parva	parvin α	Regulator of cellular adhesion	2.23
Pmm2	phosphomannomutase 2	Involved in glycoprotein biosynthesis	2.23
Cdkn2aipnl	CDKN2A interacting protein N-terminal like	Unknown function	2.22
Ndrg1	N-myc downstream regulated gene 1	Involved in stress response and cell differentiation	2.22
Angptl2	angiopoietin-like 2	Induces sprouting in endothelial cells	2.22
Sox4	SRY (sex determining region Y)-box 4	Transcriptional activator that binds to T-cell enhancer motifs	2.22
Arfip1	ADP-ribosylation factor interacting protein 1	Arf1 target protein	2.22
Dlg3	discs, large homolog 3	Regulator of synaptic plasticity	2.22
Rfk	riboflavin kinase	Involved in utilization of vitamin B2	2.22
Ppp3r1	protein phosphatase 3, regulatory subunit B, α isoform	Regulator of calmodulin stimulated protein phosphatase	2.21
Vps4a	vacuolar protein sorting 4	Involved in intracellular protein trafficking	2.21
RT1-A2	RT1 class Ia, locus A2	Antigen presentation	2.21

Map4k5	mitogen-activated protein kinase 5	Involved in transducing cell stress signals	2.21
Ppp4c	protein phosphatase 4, catalytic subunit	Phospatase regulating several cellular processes	2.21
Tra2a	transformer 2α homolog	Regulator of pre-mRNA splicing	2.211
Galnt4	N-acetylgalactosaminyltransferase 4	Catalyses initial reaction in O-linked oligosaccharide biosynthesis	2.21
Arl6ip5	ADP-ribosylation-like factor 6 interacting protein 5	Regulates intracellular concentrations of taurine and glutamate	2.21
Casp8	caspase 8	Initiator caspase mediating apoptosis	2.20
Pcsk5	Pro-protein convertase subtilisin/kexin type 5	Involved processing multiple pro-proteins to their mature forms	2.20
Dcn1	defective in cullin neddylation 1, domain containing 1	Ubiquitin-protein ligase	2.20
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	Regulator of cytokinesis and adhesion	2.20
Hdac1	histone deacetylase 1	Regulator of cell-cycle and development	2.20
Dnajc5	DnaJ (Hsp40) homolog, subfamily C, member 5	Involved in membrane trafficking and protein folding	2.20
Ghr	growth hormone receptor	Involved in post-natal tissue development	2.20
Pkia	protein kinase (cAMP-dependent, catalytic) inhibitor α	Regulator of intracellular signalling	2.20
Epas1	endothelial PAS domain protein 1	Involved in the induction of oxygen regulated genes	2.20
Elmod2	ELMO/CED-12 domain containing 2	Positive regulator of interferon response	2.19
Hpcal1	hippocalcin-like 1	Involved in calcium-dependent regulation of rhodopsin phosphorylation	2.19
Ppp2r5e	protein phosphatase 2, regulatory subunit B', epsilon isoform	Negative regulator of cell growth	2.19
LOC363060	similar to RIKEN cDNA 1600029D21	Unknown function	2.19
Kcne3	potassium voltage-gated channel, Isk-related subfamily, gene 3	Involved in epithelial electrolyte transport	2.19
Gsr	glutathione reductase	Involved in cellular antioxidant defence	2.19
Csnk1d	casein kinase 1Δ	Participates in Wnt signalling	2.19
Arpp19	cAMP-regulated phosphoprotein 19	Regulator of mitosis	2.18
Tubb4	tubulin 4β	Major microtubule component	2.18
Smad4	SMAD family member 4	Mediator of signal transduction by TGF-beta	2.18
Eif4g2	eukaryotic translation initiation factor 4 2γ	General repressor of translation	2.18
Ebag9	estrogen receptor binding site associated, antigen, 9	Caspase 3 activator involved in apoptosis	2.18
Aktip	AKT interacting protein	Regulator of apoptosis via interactions with Akt1	2.17
Snx11	sorting nexin 11	Involved in intracellular trafficking	2.17
Nsf	N-ethylmaleimide-sensitive factor	Involved in ER-Golgi transport	2.16
Ssbp3	single stranded DNA binding protein 3	Regulator of collagen expression	2.16
Mapk1	mitogen activated protein kinase 1	Extracellular signal regulated kinase	2.16
Arl5a	ADP-ribosylation factor-like 5A	GTP-binding protein involved in development	2.16
Heg1	HEG homolog 1	Unknown function	2.16
Shfm1	split hand/foot malformation (ectrodactyly) type 1	Involved in ubiquitin dependent proteolysis	2.16
Hsd17b6	hydroxysteroid (17-β) dehydrogenase 6	NAD-dependent oxidoreductase with broad substrate range	2.16
Ankrd12	ankyrin repeat domain 12	Inhibitor of nuclear receptor transcriptional activity	2.15
Rhoa	ras homolog gene family, member A	Regulator of membrane-actin stress fibre signal transduction	2.15
Gpd1	glycerol-3-phosphate dehydrogenase 1	Involved in lipid biosynthesis	2.15
Wdr33	WD repeat domain 33	Involved in cellular differentiation	2.15
Ldlr	low density lipoprotein receptor	Mediator of LDL endocytosis	2.15

Cdc16	cell division cycle 16 homolog	Regulator of cell-cycle	2.15
Psen1	presenilin 1	Increases cytoplasmic B-catenenin concentration during apoptosis	2.15
Lamc1	Laminin 1γ	Mediator of cellular adhesion and migration	2.15
Spink4	serine peptidase inhibitor, Kazal type 4	Gastrointestinal protease inhibitor	2.15
Isoc1	isochorismatase domain containing 1	Unknown function	2.14
Frem2	Fras1 related extracellular matrix protein 2	ECM protein involved in maintenance of epithelial integrity	2.14
Map3k3	mitogen activated protein kinase kinase kinase 3	Component of protein kinase signal cascade	2.14
Ifnar1	interferon (α, β and ω) receptor 1	Mediator of type I interferon signalling	2.14
Ube2h	ubiquitin-conjugating enzyme E2H	Catalyses covalent attachment of ubiquitin to other proteins	2.14
Rnf4	ring finger protein 4	Ubiquitin-protein ligase	2.14
Pld1	phospholipase D1	Involved in signal transduction and membrane trafficking	2.14
Add1	adducin 1α	Calmodulin binding promoter of actin-spectrin network assembly	2.13
Tsnax	translin-associated factor X	Nuclear targeting protein	2.13
Pmp22	peripheral myelin protein 22	Major component of myelin in the peripheral nervous system	2.13
Rab6a	RAB6A, member RAS oncogene family	Regulator of membrane traffic from the Golgi apparatus	2.13
Ddx21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	RNA helicase involved in ribosome synthesis and innate immunity	2.13
Csnk1g3	casein kinase 1 3γ	Participates in Wnt signalling	2.13
Pnrc2	proline-rich nuclear receptor co-activator 2	Involved in mRNA processing	2.13
Eif3a	eukaryotic translation initiation factor 3, subunit A	Involved in protein biosynthesis	2.12
Slc30a1	solute carrier family 30 (zinc transporter), member 1	Involved in zinc export	2.12
Ddx17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	RNA helicase	2.12
LOC685179	similar to SWI/SNF-related regulator of chromatin c2	Unknown function	2.12
Epb41l3	erythrocyte protein band 4.1-like 3	Unknown function	2.11
Fam46a	family with sequence similarity 46, member A	Unknown function	2.11
Dlg1	discs, large homolog 1	Involved in maintenance of cellular polarity and lymphocyte activation	2.11
Pdha1	pyruvate dehydrogenase (lipoamide) 1α	Involved in linking glycolysis and the TCA cycle	2.11
Hnf4	hepatocyte nuclear factor 4	Regulator of liver, kidney and intestinal development	2.11
Ensa	endosulfine α	Modulator of insulin secretion	2.11
Ifnar1	interferon (α, β and ω) receptor 1	Mediator of interferons alpha and beta signalling	2.11
Pafah1b1	platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	Involved in several dynein and microtubule-dependent processes	2.11
Mtpn	myotrophin	Involved in neuronal differentiation	2.10
Galnt2	N-acetylgalactosaminyltransferase 2 (GalNAc-T2)	Catalyzes initial reaction in O-linked glycosylation of mucins	2.10
Eif4h	eukaryotic translation initiation factor 4H	Involved in protein biosynthesis	2.10
Rbp4	retinol binding protein 4, plasma	Mediator of vitamin A (retinol) transport	2.01
Tcrb	T-cell receptor beta chain	Recognizes MHC bound antigens on antigen presenting cells	2.09
Rab27a	RAB27A, member RAS oncogene family	Mediates cytotoxic granule exocytosis in lymphocytes	2.09
Nrp2	neuropilin 2, transcript variant 4	Involved in transmembrane signalling	2.09
Klra17	killer cell lectin-like receptor, subfamily A, member 17	NK-cell pathogen recognition receptor	2.09

Tnks2	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2	Inhibitor of Wnt signalling	2.09
Dnajb14	DnaJ (Hsp40) homolog, subfamily B, member 14	Involved in membrane trafficking and protein folding	2.09
Grinl1a	glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A	Regulator of transcriptional activation	2.09
Mafg	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G	Transcriptional regulator	2.09
Snx18	sorting nexin 18	Involved in several stages of endocytosis	2.08
Sdccag3	serologically defined colon cancer antigen 3	May be involved in modulation of the TNF response	2.08
Glipr2	GLI pathogenesis-related 2	Involved in apoptosis and macrophage differentiation	2.08
Sec61a1	Sec61 1α subunit	Involved in assembly of membrane and secretory proteins	2.08
Slc16a1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	Lactate and pyruvate transporter	2.08
Ak2	adenylate kinase 2	Involved in energy metabolism and nucleotide synthesis	2.08
Tcea1	transcription elongation factor A (SII) 1	Involved in RNA polymerase II transcription	2.07
Eprs	glutamyl-prolyl-tRNA synthetase	Catalyzes the attachment of the cognate amino acid to the corresponding tRNA	2.07
Hyal3	hyaluronoglucosaminidase 3	ECM regulator	2.07
Senp5	Sumo1/sentrin/SMT3 specific peptidase 5	Component of the SUMO post-translational modification pathway	2.07
Arf6	ADP-ribosylation factor 6	Involved in vesicular trafficking and actin remodelling	2.07
Ubl3	ubiquitin-like 3	Unknown function	2.07
Dsta	dystonin transcript variant a	Component of adhesion junctions	2.06
Rab5b	RAB5B, member RAS oncogene family	GTPase modulating endosomal trafficking	2.06
Akt1s1	AKT1 substrate 1 (proline-rich)	Regulator of cell growth	2.06
LOC686428	similar to Emu2	Unknown function	2.06
Usf1	upstream transcription factor 1	Transcriptional regulator	2.06
Mki67ip	Mki67 (FHA domain) interacting nucleolar phosphoprotein	Involved in the cell-cycle	2.06
Vdac1	voltage-dependent anion channel 1	Mediator of cytochrome-c release during apoptosis	2.06
Akt2	v-akt murine thymoma viral oncogene homolog 2	General protein kinase	2.05
Ccl2	chemokine (C-C motif) ligand 2	Recruits monocytes, T(mem)-cells and dendritic cells to site of infection	2.05
Zdhhc3	zinc finger, DHHC-type containing 3	Regulator of cell surface stability	2.05
Gtpbp4	GTP binding protein 4	Involved in ribosomal synthesis	2.05
Zdhhc17	zinc finger, DHHC domain containing 17	Involved in endocytosis	2.05
LOC363060	similar to RIKEN cDNA 1600029D21	Unknown function	2.05
LOC366300	hypothetical LOC366300	Unknown function	2.04
Selt	selenoprotein T	Involved in redox regulation and cell adhesion	2.04
Rab11b	RAB11B, member RAS oncogene family	Regulator of exo/endocytosis	2.04
Arf1	ADP-ribosylation factor 1	Involved in vesicular trafficking and actin remodelling	2.04
Sf3b5	splicing factor 3b, subunit 5	Spliceosome component	2.03
Cul4b	cullin 4B	Ubiquitin-protein ligase	2.03
Nkx2-3	NK2 transcription factor related,	Possible role in cell differentiation	2.03
LOC681825	similar to Prefoldin subunit 3	Unknown function	2.02
Gna12	guanine nucleotide binding protein 12α	Membrane signal transducer	2.02

Siah1a	seven in absentia 1A	Ubiquitin-protein ligase	2.02
Camk2d	calcium/calmodulin-dependent protein kinase II delta	Transducer of calcium/calmodulin signalling	2.02
Cobl	cordon-bleu homolog	May be involved in actin modulation	2.02
Cd3d	CD3 molecule delta polypeptide	T-cell TCR/CD3 complex component mediating signal transduction	2.02
Cops4	COP9 constitutive photomorphogenic homolog subunit 4 (Arabidopsis)	Regulator of several signalling pathways	2.02
LOC288913	Similar to Leydig cell tumor 10 kD protein	Unknown function	2.02
Rcc2	regulator of chromosome condensation 2	Involved in mitosis and cytokinesis	2.02
Mrpl52	mitochondrial ribosomal protein L52	Mito-ribosomal protein component	2.01
Prlr	prolactin receptor	Hormone receptor	2.01
Jam2	junctional adhesion molecule 2	Tight junction component involved in lymphocyte homing	2.01
Smek2	SMEK homolog 2, suppressor of mek1	Regulator of microtubule organization	2.01
Prpf38b	PRP38 pre-mRNA processing factor 38 domain containing B	May be required for pre-mRNA splicing	2.01
Dusp1	dual specificity phosphatase 1	Negatively regulates mitogen-associated protein kinases (MAPK's)	2.01
Marveld2	membrane-associating domain containing 2	Integral tight junction component	2.01
Tmem20	transmembrane protein 20	Unknown function	2.01
Tbc1d1	TBC1 domain family, member 1	May regulate cell growth and differentiation	2.01
Gpkow	G patch domain and KOW motifs	Unknown function	2.01
Phf11	PHD finger protein 11	Regulator of Th1-type cytokine expression	2.01
Sema4g	sema domain, Ig, transmembrane and short cytoplasmic domain 4G	Axon guidance ligand	2.01
Foxa2	forkhead box A2	Transcription factor involved in development	2.00
Dffb	DNA fragmentation factor, β polypeptide	Pro-apoptotic caspase activated Dnase	2.00
Arih1	ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1	Ubiquitin-protein ligase	2.00
Cdc42bpb	CDC42 binding protein kinase beta (DMPK-like)	CDC42 effector involved in cytoskeletal organization	2.00
Itga6	integrin 6α	Involved in cell adhesion and cell-surface signalling	2.00

Table B4: *Genes down-regulated twofold or greater in the neonatal rat GI tract 12 h after feeding E. coli A192PP to P9 pups*

Gene Symbol	Description	Function	Mean-fold change
Nucks1	nuclear casein kinase and cyclin-dependent kinase substrate 1	May be involved in cell proliferation	-11.81
Afp	α-fetoprotein	Major plasma protein	-11.71
Tmsb10	thymosin 10β	Inhibitor of actin polymerization	-8.62
RT1-Db1	RT1 class II, locus Db1	Antigen presentation	-7.46
Adfp	Adipose differentiation related protein	Involved in sequestering lipids	-6.33
RT1-A	RT1 class I, locus A	Antigen presentation	-5.85
Cav2	caveolin 2	Involved in signal transduction	-5.00
Epsti1	epithelial stromal interaction 1	Unknown function	-5.00
Pacsin1	protein kinase C and casein kinase substrate in neurons 1	May be involved in vesicle transport	-4.95
LOC100362483	H2-GS14-2 antigen	RT1 homologue	-4.67

Tbc1d20	TBC1 domain family, member 20	GTPase-activator for Rab family proteins	-4.61
LOC688090	similar to RT1 class II, locus Bb	Antigen presentation	-4.42
Mfsd2	major facilitator superfamily domain containing 2	May regulate cell proliferation	-4.35
Amy2	amylase 2, pancreatic	Involved in starch hydrolysis	-4.33
Lox	lysyl oxidase	Initiator of collagen-elastin cross-linking	-4.22
Krit1	KRIT1, ankyrin repeat containing	Involved in microtubule formation and maintenance of endothelial integrity	-3.97
RGD1308772	similar to KIAA0564 protein	Unknown function	-3.89
Ptpn3	protein tyrosine phosphatase, non-receptor type 3	Regulator of cell adhesion	-3.88
Smarce1	SWI/SNF related regulator of chromatin e1	Regulator of transcription via chromatin remodelling	-3.66
Rsu1	Ras suppressor protein 1	Suppressor of Ras mediated signalling	-3.61
Galnt1	N-acetylgalactosaminyltransferase 1 (Galnt1), transcript variant 2	Catalyzes initial reaction in O-linked glycosylation of mucins	-3.52
Tgfb2	transforming growth factor 2β	Suppressor of IL-2 mediated T-cell growth	-3.51
8430427H17Rik	RIKEN cDNA 8430427H17 gene	Unknown function	-3.47
Ints7	integrator complex subunit 7	Involved in processing small nuclear RNA's	-3.42
Mtmr1	myotubularin related protein 1	May be involved in signalling	-3.41
Tlk2	tousled-like kinase 2	Involved in cell cycle regulation	-3.41
Phlda3	pleckstrin homology-like domain, family A, member 3	Repressor of Akt signalling	-3.40
Nr3c1	nuclear receptor subfamily 3, group C, member 1	Regulator of trans-nuclear membrane signalling	-3.25
Srp54a	signal recognition particle 54a	May mediate targeting to the ER	-3.25
Zfp191	zinc finger protein 191	Transcriptional repressor involved in development	-3.17
Wwc1	WW and C2 domain containing 1	Regulator of proliferation and apoptosis	-3.16
Pim1	proviral integration site 1	Involved in cell proliferation	-3.14
Ass1	argininosuccinate synthetase 1	Involved in arginine biosynthesis	-3.13
Ahi1	Abelson helper integration site 1	Involved in neuronal development	-3.11
Ttc21b	tetratricopeptide repeat domain 21B	Negative modulator of sonic hedgehog signal transduction	-3.09
Zfp422	zinc finger protein 422	Transcriptional regulator	-3.09
Stox2	storkhead box 2	Involved in development	-3.07
CP-2	Cyclic Protein-2	Involved in iron transport	-3.06
Tcf712	transcription factor 7-like 2, T-cell specific, HMG-box	Transcription factor involved in Wnt signalling	-3.05
Eml4	echinoderm microtubule associated protein like 4	May modify assembly dynamics of microtubules	-3.04
Znf503	zinc finger protein 503	Transcriptional repressor	-3.03
Stard3	StAR-related lipid transfer (START) domain containing 3	Cholesterol transporter	-2.99
Dlst	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	Involved in fatty acid metabolism	-2.96
Id4	Inhibitor of DNA binding 4	Regulator of DNA binding	-2.95
Pafah1b1	platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	Involved in cytoskeletal organization	-2.95
Cyfip1	cytoplasmic FMR1 interacting protein 1	Mediator of translational repression	-2.94
Mrp194	mitochondrial ribosomal protein L49	Component of the mitochondrial ribosome	-2.94
Plcxd2	phosphatidylinositol-specific phospholipase C, X domain containing 2	Involved in signal transduction	-2.93
Dpep1	dipeptidase 1	Hydrolysis of dipeptides	-2.91

Crim1	Cysteine-rich transmembrane BMP regulator 1 (chordin like)	May play a role in angiogenesis	-2.89
Acd	adrenocortical dysplasia homolog	Telosome component	-2.87
Tmem38b	transmembrane protein 38B	Mediator of rapid intracellular calcium release	-2.87
Gnas	Gs α subunit	Involved in signal transduction	-2.86
Vash1	vasohibin 1	Angiogenesis inhibitor	-2.85
RT1-Ba	RT1 class II, locus Ba	Antigen presentation	-2.83
Adam33	a disintegrin and metallopeptidase domain 33 (predicted)-like	May be involved in cell adhesion	-2.82
Mocs2	molybdenum cofactor synthesis 2	Involved in molybdopterin biosynthesis	-2.82
Tsc22d2	TSC22 domain family, member 2	Unknown function	-2.82
Tmem131	transmembrane protein 131	May be involved in immune response	-2.80
Hnrnpa1	heterogeneous nuclear ribonucleoprotein A1	Involved in pre-mRNA processing	-2.78
Ptprb	protein tyrosine phosphatase, receptor type, B	Regulator of angiogenesis	-2.78
Tmem14a	transmembrane protein 14A	Unknown function	-2.78
Thbs2	thrombospondin 2	Adhesive glycoprotein mediating cell adhesion to ECM	-2.75
Senp7	SUMO1/sentrin specific peptidase 7	Catalyses the removal of SUMO protein markers	-2.73
Slc30a2	solute carrier family 30 (zinc transporter), member 2	Zinc transporter	-2.71
Neu1	sialidase 1 (lysosomal sialidase)	Catalyzes the removal of sialic acids from proteins	-2.70
Rab30	RAB30, member RAS oncogene family	Golgi-associated signalling protein	-2.69
Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	May play a role in adaptive response to chemical exposure	-2.69
Capn7	calpain 7	Ubiquitous calcium regulated protease	-2.68
Pik3r2	phosphoinositide-3-kinase, regulatory subunit 2β	Adaptor mediating association of activated kinases to the plasma membrane	-2.67
Ube2cbp	ubiquitin-conjugating enzyme E2C binding protein	Ubiquitin-protein ligase	-2.67
Sgcb	sarcoglycan, beta (dystrophin-associated glycoprotein)	Involved in anchoring F-actin to the ECM	-2.67
Mcpt3	mast cell peptidase 3	Serine endopeptidase	-2.65
Dcaf10	DDB1 and CUL4 associated factor 10	Involved in ubiquitin-protein ligation	-2.65
Slc30a7	solute carrier family 30	Regulator of zinc homeostatis	-2.65
Slc20a1	solute carrier family 20 (phosphate transporter), member 1	Regulator of phosphate homeostatis	-2.64
Itgal	integrin Lα	Intercellular adhesion molecule receptor involved in immune cell interactions	-2.62
Ppp1r8	protein phosphatase 1, regulatory (inhibitor) subunit 8	Involved in pre-mRNA processing	-2.61
Hspa5	heat shock protein 5	Involved in regulating protein folding in the ER	-2.60
Tmem33	transmembrane protein 33	Unknown function	-2.59
Eif1b	eukaryotic translation initiation factor 1B	May be involved in translation	-2.58
Greb1	gene regulated by estrogen in breast cancer	Hormone-dependent growth regulator	-2.58
Mreg	melanoregulin	Involved in membrane fusion	-2.58
Anxa7	annexin A7	Membrane fusion promoter involved in exocytosis	-2.57
Slc34a3	solute carrier family 34 (sodium phosphate), member 3	Active phosphate importer	-2.56
Stk24	serine/threonine kinase 24	Involved in signal transduction	-2.56
Gjb3	gap junction protein, beta 3	Mediator of intercellular connexin transport	-2.55
Rnf2	ring finger protein 2	Ubiquitin-protein ligase	-2.54
Itih3	inter-α-trypsin inhibitor, heavy chain 3	Involved in binding hyaluronan to other ECM proteins	-2.53

Tmem178	transmembrane protein 178	Unknown function	-2.53
Icmt	isoprenylcysteine carboxyl methyltransferase	Involved in targeting proteins to the membrane	-2.51
Jag1	jagged 1	Notch receptor ligand and mediator of Notch signaling	-2.49
Mmp15	matrix metallopeptidase 15	Peptidase that degrades ECM components	-2.48
RGD1359529	similar to chromosome 1 open reading frame 63	Unknown function	-2.48
Romo1	reactive oxygen species modulator 1	Induces ROS production to stimulate cell proliferation	-2.46
Spsb4	splA/ryanodine receptor domain and SOCS box containing 4	Involved in ubiquitin-protein ligation	-2.46
Lpin2	lipin 2	Regulator of fatty acid metabolism	-2.44
Mcoln1	mucolipin 1	Regulator of endo/exocytosis	-2.44
Kif26a	kinesin family member 26A	Modulator of enteric neuronal development	-2.43
Dedd	death effector domain-containing	Modulator of Caspase 3 activity	-2.43
Tcirg1	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3	Proton channel involved in T-cell activation	-2.43
Idh2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	Involved in energy production and metabolism	-2.42
Arhgef10	Rho guanine nucleotide exchange factor 10	Involved in development	-2.42
Med13	mediator complex subunit 13	Co-activator of RNA polymerase II transcription	-2.41
Pign	phosphatidylinositol glycan anchor biosynthesis, class N	Involved in GPI-anchor biosynthesis	-2.41
Smad5	SMAD family member 5	Transcriptional modulator	-2.41
Procr	protein C receptor, endothelial	Involved in protein C-mediated blood coagulation	-2.40
Znf618	zinc finger protein 618	May be involved in transcriptional regulation	-2.40
Acot2	Acyl-CoA thioesterase 2	Regulator of intracellular fatty acid levels	-2.39
Tcf4	transcription factor 4, transcript variant 1	Involved in cellular differentiation	-2.39
Hsdl2	hydroxysteroid dehydrogenase like 2	Unknown function	-2.39
Ankrd28	ankyrin repeat domain 28	Involved in regulating TNF-alpha induced NFκB activation	-2.38
LOC687609	similar to ras homolog gene family, member f	Unknown function	-2.38
Ndufa5	NADH dehydrogenase (ubiquinone) 1α subcomplex 5	Involved in respiratory chain	-2.38
Park7	Parkinson disease (autosomal recessive, early onset) 7	Redox sensitive chaperone	-2.38
Smc2	structural maintenance of chromosomes 2	Involved in DNA repair	-2.37
Malat1	metastasis associated lung adenocarcinoma transcript 1	Non-protein coding regulator of cell motility	-2.36
LOC100364467	rCG36634-like	Unknown function	-2.34
LOC682058	similar to nucleolar protein with MIF4G domain 1	Unknown function	-2.33
Fam64a	family with sequence similarity 64, member A	Unknown function	-2.33
Mnt	max binding protein	Regulator of cell growth	-2.33
Pbx1	pre B-cell leukemia transcription factor 1	Transcriptional regulator	-2.33
Wfdc3	WAP four-disulfide core domain 3	Protease inhibitor	-2.32
Mllt10	myeloid (trithorax) homolog 10	Involved in tanscriptional regulation	-2.31
Thsd4	thrombospondin, type I, domain containing 4	Promotes ECM assembly	-2.31
Adar	adenosine deaminase, RNA-specific	Positive regulator of IL-2 expression in T-cells	-2.30
Cdk7	cyclin-dependent kinase 7	Regulator of cell cycle progression	-2.30
RGD1305457	similar to RIKEN cDNA 1700023M03	Unknown function	-2.30

RGD1565983	similar to apurinic/apyrimidinic endonuclease 2	Unknown function	-2.30
Spon2	spondin 2, extracellular matrix protein	Bacterial LPS binding ECM component that functions as opsonin for macrophages	-2.30
Tulp4	tubby like protein 4	Ubiquitin-protein ligase component	-2.30
Zbtb4	zinc finger and BTB domain containing 4	May be involved in transcriptional regulation	-2.30
Zfp347	zinc finger protein 347	Unknown function	-2.29
Klf5	Kruppel-like factor 5	Transcriptional regulator	-2.28
Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1	Involved in lipid biosynthesis	-2.28
Foxn3	forkhead box N3	Transcriptional repressor responding to DNA damage	-2.28
Hgd	homogentisate 1, 2-dioxygenase	Involved in amino acid catabolism	-2.28
Dapk3	death-associated protein kinase 3	Regulator of apoptosis	-2.27
Terf2	telomeric repeat binding factor 2	Regulator of telomeric stability	-2.27
Neurl1a	neuralized homolog 1A	Unknown function	-2.26
Kctd5	potassium channel tetramerisation domain containing 5	Ubiquitin ligase substrate adapter	-2.25
Tcfe3	transcription factor E3	Activator of T-cell CD40L expression	-2.25
Eif2b3	eukaryotic translation initiation factor 2B, subunit 3γ	Involved in protein biosynthesis	-2.25
Ada	adenosine deaminase	Positive regulator of T-cell co-activaton	-2.24
Slu7	SLU7 splicing factor homolog	Involved in pre-mRNA splicing	-2.24
Timp2	tissue inhibitor of metalloproteinase 2	ECM Protease inhibitor	-2.24
Tubgcp2	tubulin, gamma complex associated protein 2	Involved in tubulin assembly	-2.24
Cubn	cubilin (intrinsic factor-cobalamin receptor)	Co-transporter involved in iron metabolism	-2.23
Asxl1	additional sex combs like 1	Involved in development	-2.23
Abcc2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Mediator of bile secretion	-2.22
Hsf1	heat shock transcription factor 1	Activates heat shock responsive genes	-2.21
Nubp1	nucleotide binding protein 1	Involved in cytosolic Fe/S protein assembly	-2.21
Pnpla6	patatin-like phospholipase domain containing 6	Regulator of neuronal differentiation	-2.21
F8	coagulation factor VIII, procoagulant component	Involved in blood coagulation	-2.21
Pigy	phosphatidylinositol glycan anchor biosynthesis, class Y	Initiator of GPI anchor biosynthesis	-2.20
Atad2	ATPase family, AAA domain containing 2	Involved in cell proliferation	-2.19
Osbpl3	oxysterol binding protein-like 3	Intracellular lipid receptor	-2.19
Vtl1a	vesicle transport through interaction with t-SNAREs 1B-like	Mediator of vesicle transport pathways	-2.19
Ccdc109a	coiled-coil domain containing 109A	Unknown function	-2.18
Rnf216	ring finger protein 216	Co-activator of Il-1 induced NFB activation	-2.18
Smap1	stromal membrane-associated protein 1	Involved in clathrin-dependent endocytosis	-2.18
Npas2	neuronal PAS domain protein 2	Transcriptional regulator	-2.17
Smg7	Smg-7 homolog, nonsense mediated mRNA decay factor	Involved in nonsense-mediated mRNA decay	-2.17
Ard1a	ARD1 homolog A, N-acetyltransferase	Mediator of n-α acetylation of proteins	-2.17
Timp1	TIMP metallopeptidase inhibitor 1	ECM Protease inhibitor	-2.17
Clta	clathrin, light chain (Lca)	Mediator of endocytosis	-2.16
Mudeng	MU-2/AP1M2 domain containing, death-inducing	May be involved in apoptosis	-2.16

Pls3	plastin 3 (T-isoform)	Actin bundling protein in microvilli	-2.16
Smurf1	SMAD specific E3 ubiquitin protein ligase 1	Ubiquitin-protein ligase	-2.16
LOC680155	hypothetical protein LOC680155	Unknown function	-2.16
Dnajb5	DnaJ (Hsp40) homolog, subfamily B, member 5	May be involved in protein folding and transport	-2.15
Ptpn12	protein tyrosine phosphatase, non-receptor type 12	Signalling molecule involved in cell motility	-2.15
Mall	mal, T-cell differentiation protein-like	Involved in raft-mediated membrane trafficking	-2.15
Ubxn2b	UBX domain protein 2B	Involved in maintenance of ER and Golgi	-2.15
Nudt11	nudix (nucleoside diphosphate linked moiety X)-type motif 11	May play a role in signal transduction	-2.14
C8g	complement component 8, γ polypeptide	Component of the membrane attack complex	-2.14
Slc38a7	solute carrier family 38, member 7	Amino acid transporter	-2.14
Atxn2	ataxin 2	Unknown function	-2.13
Tgfb1i1	transforming growth factor 1β induced transcript 1	Regulator of Tgfb and Wnt signalling pathways	-2.13
Slc5a12	solute carrier family 5, member 12	Mediator of transport of monocarboxylates from intestinal lumen	-2.12
Bat5	HLA-B associated transcript 5	May be involved in immune response	-2.12
Acot1	acyl-CoA thioesterase 1	Regulator of intracellular acyl-CoA's	-2.11
LOC681665	similar to integrator complex subunit 6 isoform a	Unknown function	-2.11
Ipo11	importin 11	Receptor for nuclear localization signals	-2.11
Rnf114	ring finger protein 114	Involved in chromatin remodelling	-2.11
Ncapd2	non-SMC condensin I complex, subunit D2	Involved in protein degradation	-2.11
Psmc6	proteasome (prosome, macropain) 26S subunit, ATPase, 6	Steroid hormone receptor	-2.10
Paqr8	progestin and adipoQ receptor family member VIII	Regulator of microtubule interactions	-2.09
Ppp4r2	protein phosphatase 4, regulatory subunit 2	Unknown function	-2.09
Zfp445	zinc finger protein 445	Receptor for various ECM components	-2.08
Itgb3	integrin beta 3	Ubiquitin-protein ligase	-2.08
Ube2q1	ubiquitin-conjugating enzyme E2Q (putative) 1	Involved in microtubule-dependent cell motility	-2.08
Hdac6	histone deacetylase 6	Unknown function	-2.07
Fam82a1	family with sequence similarity 82, member A1	May be involved in mRNA splicing	-2.07
Luc7l	LUC7-like	Involved in T-cell receptor and leptin receptor signaling	-2.07
Khdrbs1	KH domain containing, RNA binding, signal transduction associated 1	Stabilizes actin cytoskeleton	-2.07
Tpm3	tropomyosin 3γ	Regulates stabilization of actin filaments	-2.07
Fam24a	family with sequence similarity 24, member A	Unknown function	-2.06
Inppl1	inositol polyphosphate phosphatase-like 1	Regulator of actin cytoskeleton remodelling	-2.06
Ptprc	protein tyrosine phosphatase, receptor type, C	Positive regulator of T-cell co-activaton	-2.06
Terf1	telomeric repeat binding factor (NIMA-interacting) 1	Involved in telomeric regulation	-2.06
Mrpl51	mitochondrial ribosomal protein L51	Component of the mitochondrial ribosome	-2.05
Sfrs14	splicing factor, arginine/serine-rich 14	May play a role in mRNA splicing	-2.05
Psd3	pleckstrin and Sec7 domain	Unknown function	-2.05
Slc30a3	solute carrier family 30 (zinc transporter), member 3	Zinc transporter	-2.05
Speg	SPEG complex locus	Regulator of cytoskeletal development	-2.05

Abhd12	abhydrolase domain containing 12	Unknown function	-2.04
Acbd3	acyl-Coenzyme A binding domain containing 3	Involved in maintenance of Golgi	-2.04
Adarb1	adenosine deaminase, RNA-specific, B1	Involved in RNA editing	-2.04
Cela2a	chymotrypsin-like elastase family, member 2A	Elastin (ECM component) specific protease	-2.04
Ints1	integrator complex subunit 1	Involved in small nuclear RNA processing	-2.04
Itpkc	inositol 1,4,5-trisphosphate 3-kinase C	Involved in nuclear export/import	-2.04
Rcn1	Reticulocalbin 1, EF-hand calcium binding domain	Regulator of Ca-dependent activities in the ER	-2.04
Clcn5	chloride channel 5	Mediator of acidification of endosomal lumen	-2.04
Abo	ABO blood group	Blood group antigen protein	-2.03
Ankrd16	ankyrin repeat domain 16	Unknown function	-2.03
Mt2A	metallothionein 2A	Heavy metal responsive protein	-2.03
Retsat	retinol saturase (all trans retinol 13,14 reductase)	May be involved in vitamin A metabolism	-2.03
Slc4a10	solute carrier family 4, sodium bicarbonate co-transporter-like, member 10	Regulator of intracellular pH	-2.03
Luc7l3	LUC7-like 3	Involved in mRNA splicing	-2.02
Mfsd7b	major facilitator superfamily domain containing 7B	Heme transporter	-2.02
Cox4i1	cytochrome c oxidase subunit IV isoform 1	Involved in mitochondrial respiratory chain	-2.02
Hunk	hormonally up-regulated neu tumor-associated kinase	Unknown function	-2.02
Mbnl1	muscleblind-like 1	Mediator of pre-mRNA splicing	-2.02
Scaper	S-phase cyclin A-associated protein in the ER	Regulator of cell cycle progression	-2.02
Serf2	small EDRK-rich factor 2	Unknown function	-2.02
Chst3	carbohydrate (chondroitin 6/keratan) sulfotransferase 3	May play a role in maintenance of T-cells	-2.02
Eftud2	elongation factor Tu GTP binding domain containing 2	Involved in pre-mRNA splicing	-2.02
Maf	v-maf AS42 oncogene homolog	Developmental regulator	-2.02
Brd1	bromodomain containing 1	Unknown function	-2.01
Cdc45l	CDC45 cell division cycle 45-like	Involved in DNA replication	-2.01
Rbbp5	retinoblastoma binding protein 5	Regulator of cell proliferation	-2.01
Traf6	Tnf receptor-associated factor 6	NFκB signal transducer	-2.01
Gnb1	guanine nucleotide binding protein (G protein), beta polypeptide 1	Modulator of transmembrane signalling systems	-2.01
Mtmr12	myotubularin related protein 12	Unknown function	-2.00
Slbp	stem-loop binding protein	May be involved in cell cycle	-2.00
A2ld1	AIG2-like domain 1	Involved in protein degradation	-2.00
Aqp7	aquaporin 7	Water/glycerol channel	-2.00
Mfap3	microfibrillar-associated protein 3	Unknown function	-2.00

Table B5: *Up- and down-regulated genes shared between P2 and P9 data sets*

Gene Symbol	Description	Function	Mean-fold change[1]
RT1-Aw2	RT1 class Ib, locus Aw2	Antigen presentation	17.7/4.00
Cirbp	cold inducible RNA binding protein	Positive regulator of cellular stress response	5.04
Iap3	Inhibitor of apoptosis 3	Apoptotic suppressor	3.91/2.52
LOC81816	hypothetical protein LOC81816	Putative ubiquitin conjugating enzyme	3.39
Rbm9	RNA binding motif protein 9	Regulates splicing of tissue specific exons	2.74
Reg3b	regenerating islet-derived 3β	Antimicrobial peptide with C-type lectin domain	2.63
Sox4	SRY (sex determining region Y)-box 4	Transcriptional activator that binds to T-cell enhancer motifs	2.61/2.22
Pik3r1	phosphoinositide-3-kinase, regulatory subunit 1α	Adaptor mediating association of activated kinases with plasma membrane	2.60/2.74
Hoxb6	homeobox B6	Transcriptional regulator	2.58
Ankle2	ankyrin repeat and LEM domain containing 2	Unknown function	2.40
Vdac1	voltage-dependent anion channel 1	Mitochondrial membrane channel involved in apoptosis	2.38
Id3	inhibitor of DNA binding 3	Inhibitor of transcription factor DNA binding	2.29
Pafah1b1	platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	Required for proper activation of Rho GTPases and actin polymerization	2.28
Vezf1	vascular endothelial zinc finger 1	Regulation of IL-3 expression	2.23/2.36
Gatad2b	GATA zinc finger domain containing 2B	Transcriptional repressor	2.21
Rnf6	ring finger protein (C3H2C3 type) 6	Ubiquitin-protein ligase	2.20
Krt15	keratin 15	Responsible for the structural integrity of epithelial cells	2.18
Ptprs	protein tyrosine phosphatase, receptor type, S	Signalling protein involved in development	2.17
Lgr4	leucine-rich repeat containing G protein-coupled receptor 4	Orphan receptor	2.09/2.35
Rod1	ROD1 regulator of differentiation 1	Regulator of cell differentiation	2.06
Eef1a1	eukaryotic translation elongation factor 1α 1	Prompter of protein biosynthesis	2.03
Nolc1	nucleolar and coiled-body phosphoprotein 1	Involved in RNA polymerase I catalysed transcription	2.02/2.4
RT1-A3	RT1 class I, locus A3	Antigen presentation	-3.11
Mcpt3	mast cell peptidase 3	Serine endopeptidase	-2.62
RT1-Db1	RT1 class II, locus Db1	Antigen presentation	-2.46
Pbx1	pre-B-cell leukemia transcription factor	Transcriptional regulator	-2.36
Pim1	pim-1 oncogene	Signalling kinase activity	-2.35
LOC100362483	H2-GS14-2 antigen	Regulation of antigen presentation	-2.29
Wwc1	WW and C2 domain containing 1	Transcriptional activator	-2.15
Amy1 ; Amy2	amylase, alpha 1A (salivary), amylase 2, pancreatic	Hydrolase	-2.03

[1]In most cases there was complete concordance between the extent of gene modulation at P2 and P9; two values are shown when there were quantitative differences between values from the two sets of animals (P2 ranked and shown first).

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